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14. ABSTRACT

Our goal is to induce a strong CD4+ T cell response against tumor antigens by preferentially presenting endogenous tumor antigens via class II major histocompatibility complex molecules (MHC II). MHC II can present endogenous tumor antigens if expressed in the absence of Invariant chain (Ii). We have up-regulated MHCII and down regulated Ii without affecting MHC II expression in tumor cells. Using the key transcription factor class II trans-activator (CIITA) we have coordinately up-regulated all class II MHC molecules (DR, DP, DQ) and associated molecules such as the Invariant chain in a Human mammary carcinoma (MCF10). We have successfully down regulated the invariant chain in MCF10 cells, up regulated for MHC II, using retroviral vectors that express siRNAs as hairpin loops. Immuno-fluorescence shows no down regulation of MHC II molecules on the cell surface after Ii was down regulated. We will test the ability of our vaccine to present tumor antigen by observing whether these cells can stimulate HER2/neu restricted CD4+ or CD8+T cells. These tumor cells could be used as a vaccine stimulating both CD4+ and CD8+ T cells in close proximity inducing a powerful long-term immune response against tumor sharing common tumor antigen with the vaccine.

15. SUBJECT TERMS
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Introduction:

The immune system is able to mount a response to tumors. The adaptive arm of the Immune system is adept at differentiating between normal (non-malignant cells) and cancerous cells due to subtle differences in the cells repertoire of peptides (1). Unfortunately many patients die even while immune cells are mounting a response to tumor. Therefore, many groups are applying various strategies to amplify any tumor specific immune response. Activation of tumor specific $CD4^+$ T cells (helper T cells) has shown to improve tumor specific immune responses and $CD8^+$ T cell (cytotoxic) memory (2). Key players in the adaptive immune response known as professional antigen presenting cells (APC) take up peptides and cross present them to $CD4^+$ T cells(3). These peptides are bound to the peptide binding groove of class II Major Histocompatibility Molecules (MHC II) which traffic to the cell surface and bind to specific T cell receptors which then, in the proper context, become activated. These activated T cells clonally expand and secrete cytokines that influence trafficking and activation of other cellular components of the immune system. In an attempt to facilitate the presentation of a greater repertoire of tumor antigens Suzanne Ostrand-Rosenberg *et. al*. have developed a method of modifying the tumor itself to present a broader repertoire of its own endogenous antigens via the MHCII system(4-16). By modifying these cells to express MHC II but not the class II accessory molecule called the invariant chain (Ii), the MHC II antigen processing and presentation is modified to better present endogenously produced antigens. The repertoire of antigens presented in the absence of Ii is different than those presented with Ii. In normal circumstances Ii is always present when MHC II is expressed. We propose that the antigens presented in the absence of Ii are unique antigens previously not "seen" by the host immune system and therefore they may activate a set of T cells that have not been previously tolerized. Tipping the balance in favor of a robust immune response to tumor antigens, to which a patient has not been tolerized, may lead to better immunotherapy for patients with existing tumors and help prevent metastasis.

Body:

Statement of Work (SOW)

Technical Objective 1: Develop siRNA for Ii and test its ability to down-regulate Ii in human and mouse breast cancer cells that constitutively express MHC II or are induced by IFNγ to express MHC II.

Task 1*: Months 1-2: Use flow cytometry to ascertain that the human mammary carcinoma cell line SUM159 and the mouse mammary carcinoma 4T1 express MHC class II when induced with IFNγ.*

Accomplished. 4T1 data unpublished; SUM159 published (15). We have also modified 4T1 to express the class II transactivator (CIITA) as a second method for upregulating MHCII and Ii (Unpublished Data).

Task 2*: Months 2-4: Design 21bp DNA oligos complimentary to 15 different sites on the human invariant chain (Ii) sense and antisense strands. Anneal the strands together and make siRNA in vitro with T7 RNA polymerase.*

Accomplished. Described in 2005 progress report and Thompson et al 2005, Appendix II (22).

Task 3*: Months 3-8: Test individual siRNAs for effective down-regulation of Ii in breast cancer cell lines described in Task 1.*

Accomplished. Using our small hairpin RNAs we can successfully down-regulate Ii by over 95%. Described in 2005 progress report and Thompson et al 2005, Appendix II. (22)

Task 4*: Months 3-8: Clone human CD80 cDNA into pLPCX retroviral vector (Clontech).*

Accomplished. Described in 2005 progress report and Dissanayake et al 2004, Appendix IV (15).

Task 5*: Months 6-10: Clone human U6 RNA Polymerase III promoter with a multi-cloning site downstream into retroviral vector pLPCX/CD80 from Task 4.*

Accomplished. (unpublished). Due to promoter interference from the 3'LTR of the retroviral vector we found that the U6 promoter was inoperable in this vector. We opted to use a commercially available retroviral vector pSIREN Retro-Q siRNA expression vector with the U6 promoter, which has the promoter function of the 3'LTR eliminated (Clontech, Carlsbad CA) (22).

Task 6*: Months 8-12: Clone DNA oligonucleotide coding for the sense-loop-antisense of successful siRNAs downstream of U6 promoter in pLPCX/CD80/U6 from Task 5. Clone DNA oligonucleotide coding for control siRNA (Lamin A/C) described by Paul et al (Paul, 2002 #23).*

Accomplished. Using our small hairpin RNA for three different siRNA sequences (32, 48 and 53) we can successfully down-regulate Ii by over 95%. When expressed by the stable retroviral vector we can stably down regulate Ii indefinitely (22).

Task 7*: Months 10-14: Transfect packaging cell line (293T Human Embryonic Kidney cells) with pLPCX/CD80/Ii^{-} retroviral vector from Task 6. Harvest virus and titer on NIH3T3 mouse fibroblasts.*

Accomplished using our pSIREN Retro-Q/hIi32 (22).

Task 8*: Months 13-16: Transduce human SUM159 and mouse 4T1 mammary carcinoma cells with retrovirus encoding CD80 and siRNA for Ii and select transductants by drug selection. Limit dilution clone if necessary.*

Accomplished using MCF10CA1 (malignant epithelial mammary carcinoma) (22). The MCF10CA1 cell line may be more indicative of a breast cancer than SUM159. MCF10CA1 cells are derived from the non-malignant MCF10A cell line giving a good negative control for immune response against malignant versus non-malignant phenotypes. Also MCF10CA1 is IFNγ inducible and can be up-regulated by CIITA expression for HLA-DR7 a common allele in the Caucasian population making it easy to match patient/donor peripheral blood mononuclear cells (PBMC).

A separate siRNA was designed to down regulate mouse Ii. The human siRNA could not be used because the sequence homology of the mouse and human Ii was not similar enough. The mouse siRNA were designed targeting the same region of the mouse Ii mRNA as were shown to be effective targets of siRNA for human Ii mRNA. The same small hairpin RNA expression vector used for our human siRNA was used to clone mouse siRNA for Ii. This vector has been shown to have promoter activity in both human and mouse (personal communications with Clontech, Calsbad, CA). Mouse cell lines 4TI (mammary sarcoma) and SaI (acites) were transduced with the siRNA expression vector. Mouse siRNA 54 down-regulated Ii greater than 95% as shown in figures 1 and 2, Appendix I. SaI tumor was used to confirm the functionality of the mouse Ii siRNA in another cell line.

Task 9*: Months 16-24: Induce cells generated in Task 8 with IFNγ. Use flow cytometry to verify MHC II and CD80 expression and functionality of the siRNA to down regulate Ii.*

Accomplished (22). Cells were transduced to express CIITA giving stable class II expression.

Technical Objective 2: As a model system in which to test "proof of principle" we will test the siRNA approach in vivo in mice with metastatic mammary carcinoma.

Task 10*: Months 20-24: Inoculate naïve BALB/c mice with genetically modified 4T1 cells and follow for survival and quantify number of metastatic cells in the lungs, liver, bone marrow, and brain using the clonogenic assay.*

Aborted. Preliminary experiments using the mouse SaI sarcoma cell line transfected with CIITA, CD80 and mouse Ii-siRNA expression vector were did not significantly extend survival of mice when compared with control SaI cell line (Data not shown). This data did not reflect earlier studies which showed MHC II^+ Ii^- SaI to extend survival of mice when compared with parental SaI tumor (4). The SaI/CIITA/CD80/Ii-siRNA cells were MHC II^+ and Ii^- by flow cytometry and Western analysis. The level of MHC II in the Ii-siRNA cells was lower than the MHCII gene modified cells described previously and this difference in expression levels may explain the divergence. We therefore did not pursue the lengthier 4TI experiments until we could better understand the reason why the Ii-siRNA cells failed to convey significantly greater survival in the SaI mouse model.

***Task 11**: Months 24-30: Inoculate BALB/c mice with wild type 4T1 tumor cells and allow primary tumors to metastasize. Surgically remove primary tumors and treat mice with irradiated retrovirally transduced CD80$^+$Ii$^-$ mouse 4T1 cells that have been in vitro treated with IFNγ. Follow mice for survival and quantify the numbers of metastatic cells in distant organs per task 10.*

Aborted. As described for Task 10, because the Ii-siRNA transduced mouse SaI cells failed to extend survival of mice we were unable to move forward with the 4TI experiments.

Technical Objective 3: Test MHC II$^+$ CD80$^+$ Ii$^-$ human breast cancer cells for their ability to activate breast-cancer-specific CD4$^+$ T cells from PBMC of tumor-bearing individuals.

***Task 12:** Months 18-22: Using primary tumor for which autologous PBMC are available (provided by our collaborator, Dr. A. Stopeck), identify primary breast cancer cells, which are IFNγ inducible or constitutively express MHC II.*

Task 12 modified: Because of the advantages of MCF10CA1 (Listed in the response to Task 8) we have used this cell line and partially HLA matched PBMC from healthy donors to study T cell responses to vaccine.

HLA-matched patient PBMCs are used in future experiments.

***Task 13**: Months 22-25: Transduce the primary human breast cells identified in Task 12 with the CD80$^+$ siRNA retrovirus. Induce cells with IFNγ, and use flow cytometry to ascertain expression of MHC II and CD80, and lack of Ii expression.*

Accomplished. The MCF10CA1 cell line has been transduced with CD80, CIITA, and Ii siRNA expression vector and shown by flow cytometry and western analysis to express MHC II, and CD80 but not the Ii (Appendix II) (22, 23).

***Task 14**: Months 25-32: Perform antigen presentation assays using autologous PBMCs as responding lymphocytes and retrovirally-modified autologous breast cancer cells from task 13 as antigen presenting cells. Quantify T cell activation by measuring IL-2 production by ELISA.*

Accomplished. PBMC were primed with either Tumor antigen HER2 p98, p776 or with tumor vaccine cells (MHCII$^+$, CD80$^+$, Ii-), expanded on IL-2 or IL-15 for one week then boosted with tumor vaccine or control tumor lines.

We have successfully boosted PBMC, that were primed with a known breast cancer tumor antigen HER2 (p98 and p776), with tumor vaccine (22) (Appendix II).
We have successfully primed and boosted partially matched PBMC with tumor vaccine (figure 3, Appendix II and Appendix III).

PBMC primed and boosted with Ii^- tumor vaccines consistently activated a greater $CD4^+$ T cell response when compared with Ii^+ tumor vaccines, as shown by increased IFNγ secretion (Figure 4, Appendix III). We have also shown that the responding cells in the activated PBMC are $CD4^+$ T cells, as shown by CD4 depletions (Figure 4, Appendix II & III). The vaccine cells are only MHC class II matched to the PBMC. Tumor vaccines require MHC II to activate PBMC (Figure 4, Appendix II & III).

PBMC primed with Ii^- tumors (MCF10/DR7/CD80) give significantly greater IFNγ secretion when boosted with Ii^- tumors than with Ii^+ tumors (Figure 5a and b, appendix II & III). PBMC primed with Ii^+ tumors and boosted with Ii^+ or Ii^- tumors have no significant change in IFNγ secretion (Appendix I Figure 5a,b, Appendix II & III). Further analysis of PBMC primed with Ii^+ tumor showed that these PBMC when boosted with Ii^- tumor gave significantly increased activation in many experiments (Appendix I Figure 6, Appendix II & III). This data supports other studies that show that in the absence of Ii different antigens are presented than in the presence of Ii (18). This also supports our theory that in the absence of Ii, novel tumor antigens are presented for which T cells have not previously been selected against or tolerized. This data also suggest that Ii^- tumors present many, if not all the, antigens presented in the presence of Ii. Ii^- cells present other antigens not presented by Ii^+ cells (23, appendix III). Therefore, a greater array of antigens are presented in the absence of Ii, some of which may activate a greater immune response than those presented normally in the presence of Ii.

Ii^- and Ii^+ vaccine cells activate overlapping, but distinct, repertoires of $CD4^+$ T cells
If Ii^- vaccine cells present novel tumor antigen epitopes that are not presented by Ii^+ cells, then Ii^- and Ii^+ cells should activate different repertoires of $CD4^+$ T cells. This hypothesis was tested by assessing the TCR V_β repertoires of the $CD4^+$ T cells activated by Ii^+ vs. Ii^- cells. PBMC from $DR7^+$ healthy human donors were primed and boosted with Ii^- (MCF10/DR7/CD80) or Ii^+ (MCF10/DR7/CD80/Ii) cells, and the non-adherent cells were harvested and triple-stained for CD4 antigen and 24 different TcR V_β antigens. The $CD4^+$ T cells were gated (Appendix I Fig. 7a & appendix III) and analyzed for V_β expression. Figure 7b shows the TCR V_β family usage by $CD4^+$ T cells from healthy donors 123104 and 100704 before and after activation with either the Ii^- or Ii^+ cells. Table 1 shows the number of TCR V_β families that are increased at least 20% in response to Ii^- or Ii^+ cells in these two donors plus a third healthy donor (111504). Some TCR V_β families are equally activated by both Ii^- and Ii^+ cells, while other families are activated exclusively by Ii^- or Ii^+ APC. Therefore, Ii^- and Ii^+ cells activate some of the same TCR V_β families, but they also activate distinct families, demonstrating that Ii^- vaccine cells activate $CD4^+$ T cells that are not activated by standard APC that are Ii^+.

Task 15*: Identify the responding T cells in Task 14 by blocking MHC class I or II and/or by depleting CD4 or CD8 T cells before setting up the antigen presentation assays. Ascertain the type of CD4 response (Th1 or Th2) by assessing IL-4 and IFNγ production by ELISA and in situ cytokine capture.*

Accomplished. MHC II matched, MHC I mismatched PBMC depleted of $CD4^+$ T cells are not significantly activated by $MCF10/MHCII^+$ Ii^- cells (22, 23, appendix II & III).

$CD8^+$ T cell depleted PBMC are activated by MCF10/$MHCII^+$ Ii^- cells (22, 23, appendix II & III). Therefore MHC II matched MHC II^+ Ii^- tumor cells can activate tumor specific $CD4^+$ T cells. We have shown that tumor vaccine cells induce a Th1 response as shown by high IFN-γ and low IL-4 production as ascertained by ELISA (23, appendix III).

Key Research Accomplishments:

- Development of siRNA that down regulates the mouse Invariant chain by over 95%.
- Creation of mouse mammary sarcoma cell line 4TI which expresses CIITA inducing expression of MHC II as well as Ii. These cells were further transduced with a stable small hairpin RNA expression vector which down regulates Ii by over 95% giving a mouse tumor vaccine, which is MHC II^- and Ii^-.
- Creation of a human Invariant chain vector and transduction of MCF10/DR7/CD80/Ii vector to aid in studying the effects of Ii on antigen processing and presentation and how that effects efficacy of tumor vaccines.
- Priming and boosting of donor PBMC with tumor vaccine alone, giving very high IFN-γ secretion, indicative of high T cell activation.
- Identified, through depletion of T cell subpopulations, the responding $CD4^+$ T cells to MHC II matched vaccines.
- Showed that Ii^- tumors vaccines induce a more prolific activation of $CD4^+$ T cells than Ii^- tumor vaccines.
- Showed that Ii^- tumor vaccines activate a broader range of T cells to a greater extent than Ii^- tumor vaccines.

Reportable Outcomes:

Publications:

- **James A. Thompson**, Minu K. Srivastava, Jacobus J. Bosch, Virginia K. Clements, Bruce R. Ksander, Suzanne Ostrand-Rosenberg. The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 $CD4^+$ T lymphocytes. Cancer Immunol. Immunother. 2008. 57: 389-98.
- **James A. Thompson**, Samudra K. Dissanayake, Keith L. Knutson, Mary N. Disis, and Suzanne Ostrand-Rosenberg. Tumor Cells Transduced With the MHC Class II Transactivator Activate Tumor-Specific $CD4^+$ T cells Whether or Not They are Silenced for Invariant Chain. Cancer Res. 2006 66: 1147-1154.
- Dissanayake SK, **Thompson JA**, Bosch JJ, Clements VK, Chen PW, Ksander BR, Ostrand-Rosenberg S. Activation of tumor-specific $CD4^+$ T lymphocytes by major histocompatibility complex class II tumor cell vaccines: a novel cell-based immunotherapy. Cancer Res. 2004 64:1867-1874.

- Jacobus J. Bosch, **James A. Thompson**, Minu K. Srivastava, Uzoma K. Iheagwara, Timothy G. Murray, Michal Lotem, Bruce R. Ksander, and Suzanne Ostrand-Rosenberg. *MHC II Transduced Tumor Cells Originating in the Immune Privileged Eye Prime and Boost CD4$^+$ T Lymphocytes That Cross-react with Primary and Metastatic Uveal Melanoma Cells.* Cancer Res. 2007 67: 4499-506.

Invited Oral Presentations:

- **Thompson J. A.**, Minu K. Srivastava, Jacobus J. Bosch, Bruce R. Ksander, Suzanne Ostrand-Rosenberg. The Absence of Invariant Chain in MHC II Cancer Vaccines Enhances the Activation of Tumor-Reactive Type 1 CD4$^+$ T Lymphocytes. American Association of Cancer Researchers Annual meeting 2007.
- **Thompson, J. A.**, Dissanayake S. K., Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA interference (RNAi) to down regulate invariant chain. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, Philadelphia, PA, USA; 2005
- **Thompson, J. A.** & Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA Interference (RNAi) to down regulate invariant chain: An MHC II$^+$ Ii$^-$ tumor vaccine approach. University of Maryland Graduate Student Association of Biological Sciences (GABS) Symposium, Baltimore, MD, USA; 2004

Poster presentations:

- **Thompson, J. A**., Dissanayake S. K., Ostrand-Rosenberg S., Tumor Cells Transcuced with the MHC Class II Transavtivator and Silenced for Invariant Chain Activate Tumor-Specific CD4$^+$ T Lymphocyts and are Potential Cancer Vaccines. Cancer Research Institute (CRI) Cancer Vaccines, NewYork, NY, USA; 2005
- **Thompson, J. A**., Dissanayake S. K., Ostrand-Rosenberg S., Tumor cells can stably express MHC II in the absence of the Invariant chain (Ii): RNAi down regulation of Ii does not effect surface levels of HLA-DR7. Tumor Vaccine and Cell Therapy (TVACT), Anehiem, CA, USA; 2005 & Basic Aspects of Tumor Immunology, Keystone, CO, USA; 2005
- **Thompson, J. A**., Dissanayake S. K., Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA interference (RNAi) to down regulate invariant chain. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, Philadelphia, PA, USA; 2005

Conclusions:

The human MHC II vaccines described in this report are generated by transducing Ii-MHC I^+ mammary carcinoma cells with CD80 and MHC II alleles syngeneic to the prospective recipient. Previous studies demonstrated that such vaccines induce tumor-specific immunity that results in rejection of established primary and metastatic disease in mouse models [4, 5, 7, 24]. This report extends the mouse studies to humans and demonstrates that the absence of Ii in the vaccine cells is a critical element for vaccine efficacy. We find that MHC II vaccines prime and boost type 1 $CD4^+$ T cells in vitro to release high levels of IFNγ. MHC II vaccines consistently induced greater expansion of $CD4^+$ T cells that secreted more IFNγ and expressed a different repertoire of T cell receptor gene families than $CD4^+$ T cells primed and/or boosted by the same cells expressing Ii. Collectively, these observations are consistent with the hypothesis that the absence of Ii facilitates a more robust $CD4^+$ T cell response that includes the presentation of tumor peptides that are presented by Ii^+ APC, as well as distinct peptides that are uniquely presented by Ii^- APC.

There are several possible explanations why the absence of Ii promotes greater expansion of T cells and increased IFNγ secretion by activated $CD4^+$ T cells. In contrast to professional APC, the peptide binding groove of newly synthesized MHC II molecules of MHC II vaccines is not occupied by Ii or CLIP, and may bind peptides derived from endogenously synthesized molecules either in the ER or as they traffic from the ER to endosomes. If the ER contains peptides that are not present in endosomes, then the genetically modified tumor cells may be presenting peptides that are not presented by professional APC. Such antigen presentation would result in the activation of $CD4^+$ T cells that are not typically activated by professional Ii^+ APC. Other studies using different experimental approaches support this possibility. For example, Ii^- MHC II vaccine cells activate $CD4^+$ T cells to peptides derived from diverse subcellular compartments, whereas Ii^+ APC do not [13], consistent with the concept that Ii^- MHC II vaccine cells present a different repertoire of tumor peptides than the repertoire presented by Ii^+ APC. Studies with Ii knockout mice similarly show that the absence of Ii facilitates the presentation of novel endogenous epitopes, while most endogenous antigens that are presented in the presence of Ii are also presented in the absence of Ii [19]. These findings are also corroborated by biochemical studies in which mass spectroscopy analysis of MHC II-bound peptides demonstrated that Ii^- APC display peptides that are not presented by Ii^+ APC [18].

The presentation of different and/or more immunogenic epitopes by Ii^- MHC II vaccines may also be due to the presentation of “cryptic” peptides, a term coined by investigators studying autoimmunity. Cryptic peptides are peptides that are not presented in the thymus during central tolerance induction, but are present in the periphery and which bind with high affinity to MHC molecules. Such peptides have been identified and shown to induce T cell responses to self antigens [25-28]. If the absence of Ii in MHC II vaccine cells results in altered antigen processing and presentation, as we have hypothesized, then the vaccine cells may present cryptic tumor peptides which potentially induce a more robust $CD4^+$ T cell response.

The MCF10/DR7/CD80 and MCF10/CIITA/CD80/siRNA vaccines both activate tumor reactive $CD4^+$ T cells in the absence of Ii and have the potential to be clinically useful. We have proposed using the vaccines in non-autologous patients who are HLA-DR-matched to the vaccine cells, to avoid customized vaccine preparation for individual patients [29]. Each vaccine has its pros and cons. Advantages of the multiple allele CIITA-based vaccines include a) their presentation of tumor peptides on multiple MHC II alleles; b) their ease of production via a universal construct encoding the CIITA, Ii siRNA, and CD80; c) the potential to use tumor cells that constitutively express MHC II; and d) increased expression of MHC class I molecules due to up-regulation of MHC I by the CIITA. A drawback of the CIITA vaccines is their potential to activate allo-MHC II responses, which may overwhelm the tumor-specific MHC II-restricted response in non-fully MHC II-matched recipients. A major advantage of the single allele vaccines is the ease of HLA-DR-matching of the vaccine to an individual patient. A potential disadvantage of the single allele vaccines is that an IFNγ-inducible tumor cell line could be upregulated for Ii if the vaccine enters or induces an inflammatory environoment. However, this disadvantage can be overcome if non- IFNγ-inducible cells are used or if the cells are cotransduced with Ii siRNA. Both the single allele and multiple allele vaccines could be stored as frozen cells, thereby minimizing reagent preparation time for individual patients.

Several clinical observations support our hypothesis that tumor cell co-expression of Ii with MHC II blocks tumor cell immunogenicity and favors tumor progression by impeding T cell activation to endogenously synthesized tumor antigens. Chamuleau and colleagues have observed that acute myelogenous leukemia patients whose myeloid leukemic blasts are $HLADR^+$ $CLIP^-$ or low have a significantly better clinical prognosis than patients whose blasts are DR^+CLIPhigh [30]. Since the failure to remove CLIP from MHC II molecules reduces endogenous antigen presentation [31], this observation is consistent with the concept that HLA-DR expression in the absence of Ii favors the activation of tumor-reactive $CD4^+$ T cells. Similarly, the coexpression of Ii by $HLA\text{-}DR^+$ hepatocellular carcinoma cells is associated with a very poor prognosis [32]. Likewise, tumor cell expression of an isoform of Ii that blocks endogenous antigen presentation is associated with poor prognosis in chronic lymphocytic leukemia patients [33]. If the vaccine cells are the actual APC in vivo, then they may be particularly useful for treating cancer patients who's DC are dysfunctional due to tumor burden [34, 35]. If the vaccine cells are not the relevant APC in vivo, and $CD4^+$ T cells are activated via cross-dressing [36] or by conventional antigen presentation via DC, then the vaccines are useful reagents for producing novel peptide-MHC complexes. Regardless of the precise mechanism by which the MHC II vaccines activate tumor-reactive $CD4^+$ T cells, they efficiently prime and boost $CD4^+$ T cells, and the absence of Ii is critical for their activity.

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DAMD 17-03-1-0337 Appendix I (Figures)

Figure 1

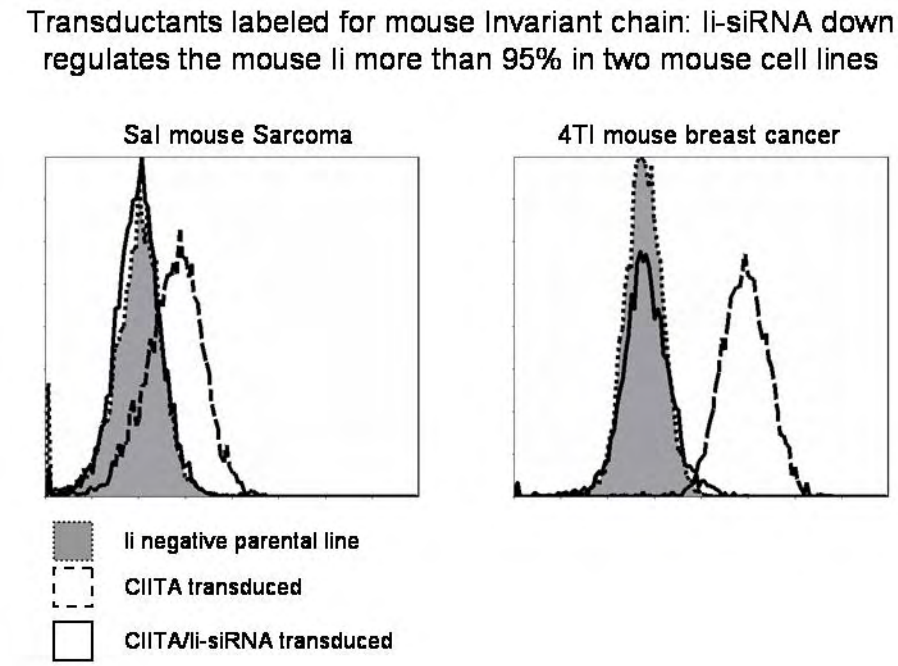
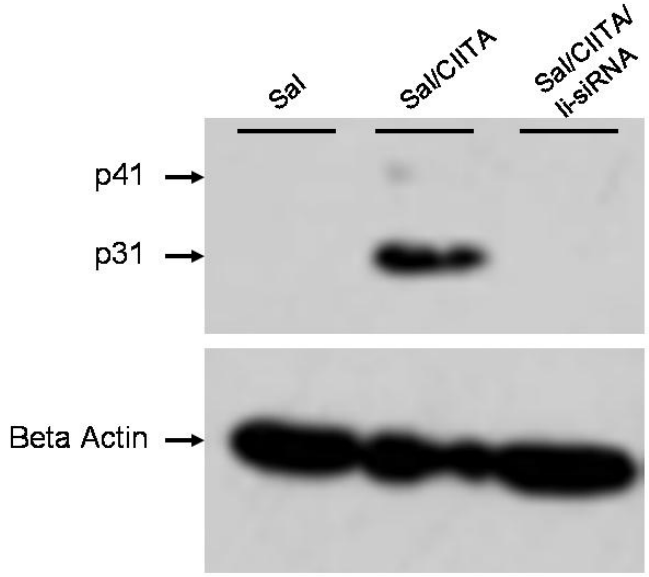


Figure 2

Mouse Ii siRNA down regulates Ii by over 95% as shown by Western analysis



Appendix I (Figures)

Figure 3

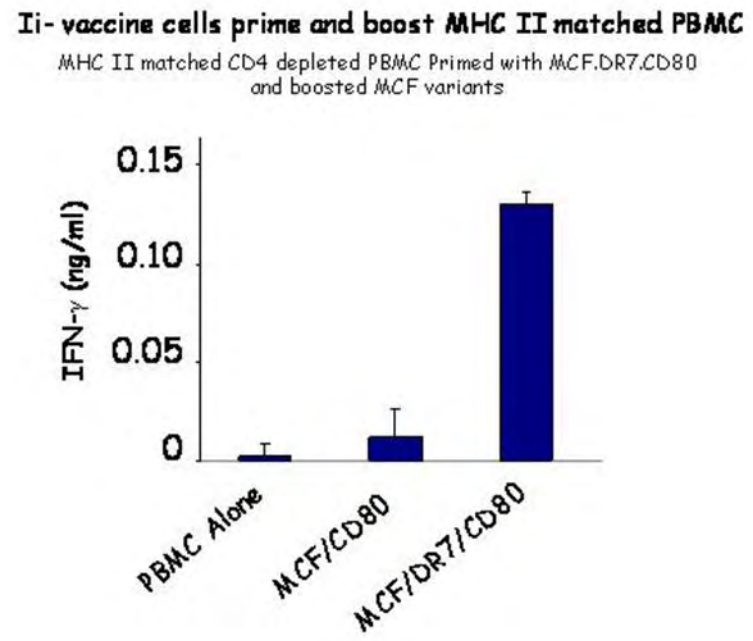
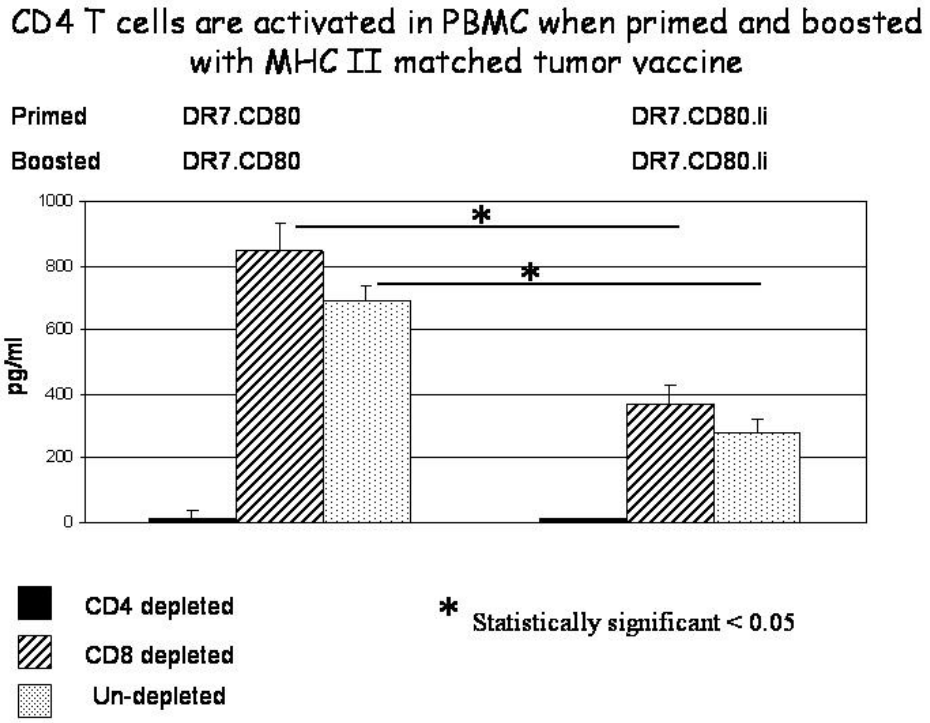


Figure 4



Appendix I (Figures)

Figure 5a

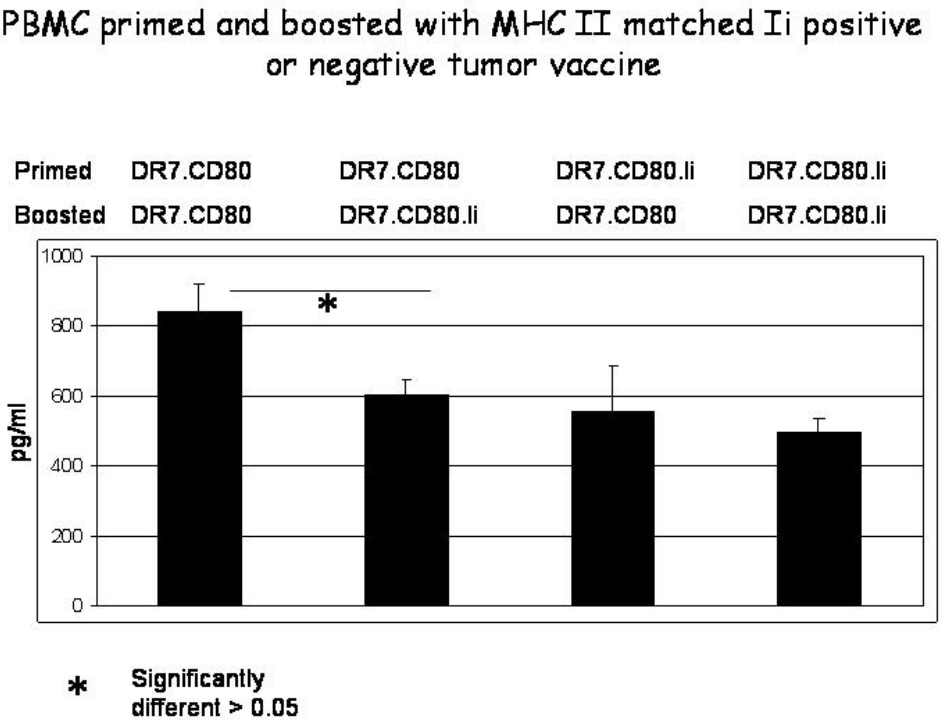


Figure 5b

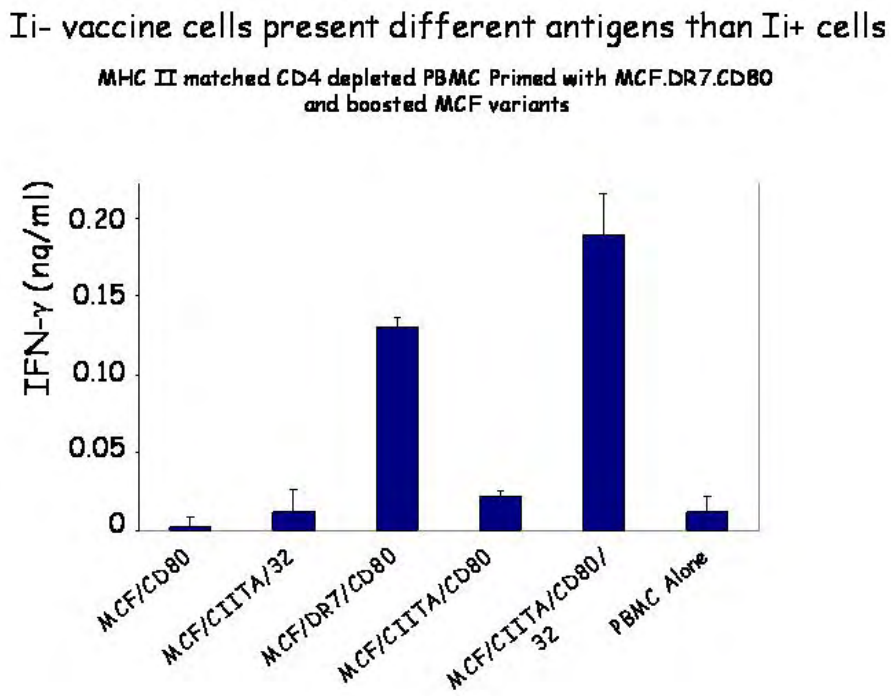
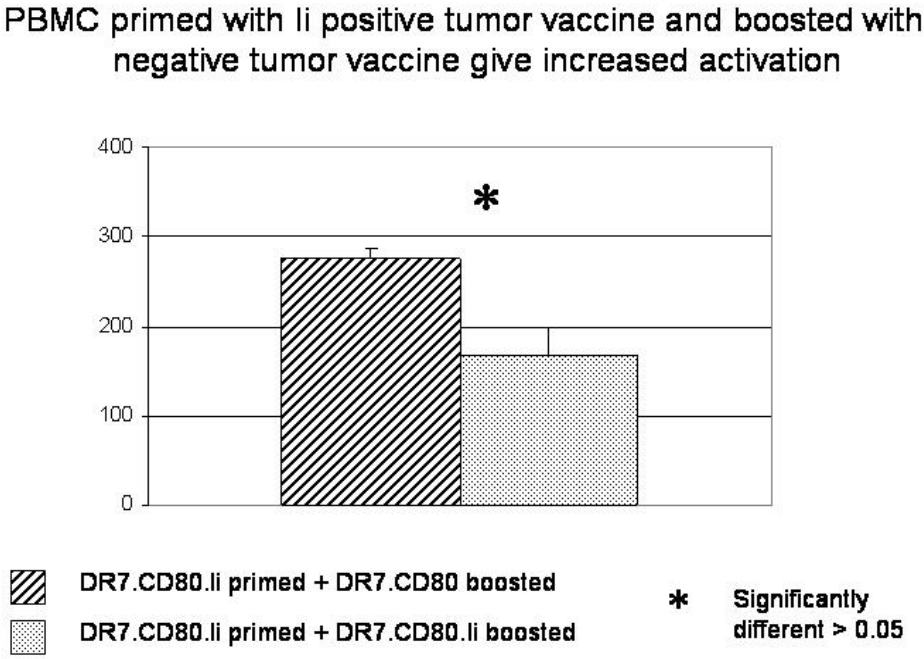


Figure 6


PBMC primed with Ii positive tumor vaccine and boosted with Ii negative tumor vaccine give increased activation
400
300
200
100
0
*
DR7.CD80.Ii primed + DR7.CD80 boosted
DR7.CD80.Ii primed + DR7.CD80.Ii boosted
* Significantly different > 0.05

Appendix I (Figures)

Table 1

Table 1. Ii^- and Ii^+ breast cancer cells activate over-lapping and distinct repertoires of $CD4^+$ T cells.

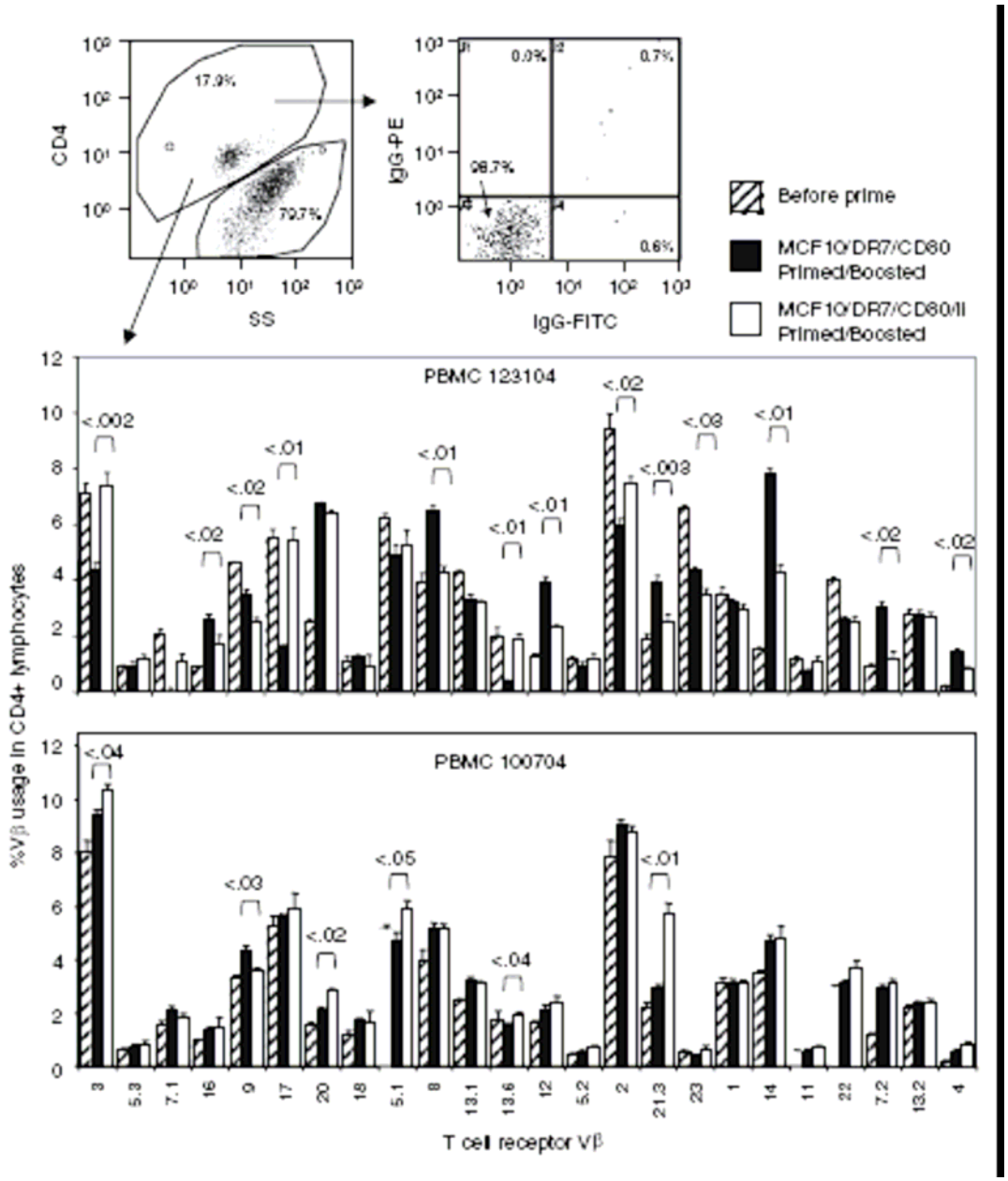
PBMC donor	Number of TCR Vβ families expanded after activation[a]		
	Ii^+ APC	Ii^- APC	Ii^+ and Ii^- APC[b]
123104	1	2	6
100704	1	3	10
111504	1	6	5

[a] Expansion of a given TCR Vβ family is defined as a statistically significant increase ($p< 0.5$) of at least 20% in the percent of $CD4^+$ T cells after priming and boosting compared to unprimed $CD4^+$ T cells.

[b] Number of common TCR Vβ families expanded by both Ii^- and Ii^+ APC

Appendix I (Figures)

Figure 7



Tumor Cells Transduced with the MHC Class II Transactivator and CD80 Activate Tumor-Specific CD4$^+$ T Cells Whether or Not They Are Silenced for Invariant Chain

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Abstract

The specificity and potency of the immune system make immunotherapy a potential strategy for the treatment of cancer. To exploit this potential, we have developed cell-based cancer vaccines consisting of tumor cells expressing syngeneic MHC class II and costimulatory molecules. The vaccines mediate tumor regression in mice and activate human CD4$^+$ T cells *in vitro*. Previous vaccines were generated by transducing MHC II negative tumor cells with a single *HLA-DR* allele. Because expression of multiple MHC II alleles would facilitate presentation of a broader repertoire of tumor antigens, we have now transduced tumor cells with the MHC class II transactivator (*CIITA*), a regulatory gene that coordinately increases expression of all MHC II alleles. Previous studies in mice indicated that coexpression of the MHC II accessory molecule invariant chain (Ii) inhibited presentation of endogenously synthesized tumor antigens and reduced vaccine efficacy. To determine if Ii expression affects presentation of MHC class II–restricted endogenously synthesized tumor antigens in human tumor cells, HLA-DR-MCF10 breast cancer cells were transduced with the CIITA, CD80 costimulatory molecule gene, and with or without small interfering RNAs (siRNA) specific for Ii. Ii expression is silenced >95% in CIITA/CD80/siRNA transductants; down-regulation of Ii does not affect HLA-DR expression or stability; and Ii$^+$ and Ii$^-$ transductants activate human CD4$^+$ T cells to DRB1*0701-restricted HER-2/*neu* epitopes. Therefore, tumor cells transduced with the CIITA, CD80, and with or without Ii siRNA present endogenously synthesized tumor antigens and are potential vaccines for activating tumor-specific CD4$^+$ T cells. (Cancer Res 2006; 66(2): 1147-54)

Introduction

Immunotherapy is a potential approach for the treatment and/or prevention of cancer because of its specificity, sensitivity, potency, and long-term memory. T lymphocytes, the cellular arm of the immune response, are particularly promising because they have the capability of localizing to tumor sites and directly killing tumor cells. Because of these characteristics, vaccines and/or immunotherapy may facilitate the destruction of existing disseminated metastatic tumor cells and protect individuals against the recurrence of primary tumors and/or the outgrowth of latent metastatic cells (1, 2).

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T cells that are cytotoxic for tumor cells are typically CD8$^+$ T lymphocytes, and optimal activation of these cells usually requires coactivation of CD4$^+$ T helper lymphocytes (3, 4). CD4$^+$ T lymphocytes are also required for generating CD8$^+$ T memory cells (5–7). Because of these critical roles for CD4$^+$ T cells, we are developing cancer vaccines that specifically target the activation of CD4$^+$ T cells while concurrently activating cytotoxic CD8$^+$ T lymphocytes.

CD4$^+$ T lymphocytes are activated to peptide antigen that is presented by MHC class II molecules. Because MHC II molecule expression is usually limited to professional antigen-presenting cells (APC), immunity to most pathogens requires that professional APCs acquire antigen from exogenous sources. To facilitate the presentation of endocytosed antigen, professional APCs contain the MHC class II–associated accessory molecule, invariant chain (Ii). Ii hinders the presentation of endogenously synthesized peptides and favors the presentation of antigen acquired by endocytosis. It mediates this effect by binding to newly synthesized MHC class II molecules in the endoplasmic reticulum and preventing them from acquiring peptides of endogenously synthesized molecules. The Ii chain also contains trafficking signals, which guide newly synthesized MHC II molecules to the endocytic pathway where Ii protein is degraded and peptides derived from endocytosed proteins bind (reviewed by refs. 8, 9). In professional APCs, MHC class II and Ii molecules are coordinately regulated at the transcriptional level by the MHC class II transcriptional activator (*CIITA*), a master regulatory gene that controls expression of all MHC II alleles (10–13). This coordinate regulation assures that professional APCs efficiently present antigenic peptides acquired from extracellular sources.

Ii has been considered essential for MHC II function. Its requirement is supported by the finding that Ii knockout mice have dysfunctional or very low levels of MHC class II molecules, and CD4$^+$ T cell activation is minimal (14–18). In contrast, some Ii-negative nonprofessional APCs when transfected or transduced with MHC class II genes present antigen and activate CD4$^+$ T cells (16, 19–22), suggesting that MHC class II molecules can be fully functional in the absence of Ii.

Based on the assumption that coexpression of Ii blocks endogenous antigen presentation, we have produced cancer vaccines by transducing MHC II tumor cells with syngeneic MHC II and costimulatory molecule genes. The vaccines mediate tumor regression in mice and activate tumor-specific CD4$^+$ T cells (20, 23–26). Activated CD4$^+$ T cells in both mouse and human systems are specific for antigens encoded by the vaccine cells. These vaccines have been produced by transducing MHC class II tumor cells with a single *HLA-DR* allele. Because expression of multiple

MHC II alleles may facilitate presentation of a broader repertoire of tumor antigens, we have now transduced tumor cells with the CIITA. To determine if Ii coexpression affects T-cell activation to endogenous antigen, we have introduced small interfering RNAs (siRNA) specific for Ii into human tumor cells transduced with the *CIITA* and *CD80* costimulatory molecule genes. The transductants efficiently activate human $CD4^+$ T cells to HER-2/*neu* tumor antigen epitopes, suggesting that this strategy may be useful for vaccine design. Properly conformed and functional MHC II heterodimers are present in transductants with or without Ii siRNA, indicating that Ii is not essential for the HLA-DR function. Surprisingly, transductants with or without the Ii siRNA are equally efficient at activating $CD4^+$ T cells, indicating that in this system, Ii does not impair endogenous antigen presentation.

Materials and Methods

Cells. SUM159PT, Jurkat, Sweig, 293T, and peripheral blood mononuclear cells (PBMC) were handled as described (20). The human breast cancer line MCF10CA1 (hereafter called MCF10) and its nonmalignant counterpart MCF10A were cultured in MCF10 medium (DMEM/Hams F12, 1:1; 5% heat-inactivated FCS; 0.029 mol/L Na bicarbonate; 10 mmol/L HEPES; ref. 27) or MCF10A medium [MCF10 medium supplemented with 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin (all from Sigma, St. Louis, MO), and 20 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA). MCF10 transductants were supplemented with puromycin (0.3 μg/mL; Clontech, Palo Alto, CA) or hygromycin (150 μg/mL; Calbiochem, San Diego, CA). The OMM2.3 human ocular melanoma line (28) was grown in RPMI with 10% heat-inactivated FCS and 5×10^{-5} mol/L β-mercaptoethanol. All cell lines and procedures were approved by the institutional review boards of the participating institutions.

siRNA. Complementary sequences in the coding region of the human *Ii* gene (Genbank accession no. NID NM_004355) were identified using the Ambion siRNA target finder search engine (Ambion, Inc., Austin, TX). Sequences with no homology to other known human mRNAs were chosen at random from the 3′ to 5′ end of the Ii mRNA. siRNAs were produced by *in vitro* transcription with T7 RNA polymerase (29) using the Ambion Silencer siRNA Construction kit and were made homologous to sequences 4, 8, 16, 24, and 50 (double adenine regions found by the Ambion target finder search engine). siRNAs were transfected into 293T/CIITA cells (20) using 40 ng siRNA. siRNAs producing 4- to 50-fold decreases in Ii expression were identified, and oligonucleotide siRNA expression cassettes were prepared by MWG Biotech (High Point, NC) and inserted into the pSIREN Retro-Q vector (Clontech) according to the manufacturer's directions (Clontech). The forward and reverse primers were annealed and ligated to the linearized pSIREN-RetroQ vector with *Bam*HI and *Eco*RI "sticky ends."

Retroviral constructs, transductions, and drug selection. The human *CIITA* gene was cloned from pcDNA1-amp/tagCIITA (30) into Litmus28 (New England Biolabs, Beverly, MA) using *Xba*I and *Eco*RI and then cloned into a modified pLNCX retroviral vector, pLNXC2(AvrII) (neo resistance; ref. 20) using *Bgl*II and *Avr*II.

The HLA-DRB1*0701 cDNA (in RSV.5 vector; ref. 31) contained two point mutations: a guanine instead of an adenine at base 13 and an adenine instead of a thymine at base 191. These errors were corrected using Splicing by Overlapping Extensions (SOEing; ref. 32) using the following four primers in successive SOEing reactions: primer 1, AGTACCCGGGATGGTGTG-TCTGAAGCTCCCTG; primer 2, AGCGCACGAACTCCTCCTGGTTATAGAA; primer 3, TTCTATAACCAGGAGGAGTTCGTGCGCT; primer 4, TAGTGCG-GCCGCTCAGCTCAGGAATCCTGTTG. Reaction 1: 10 pmol/L RSV.5/DRB1*0701 template and 0.5 μmol/L of primers 1 and 2; cycle at 95°C for 2 minutes, then 30 cycles of 95°C for 30 seconds, 60.2°C for 30 seconds, 72°C for 1 minute, then 72°C for 10 minutes. Reaction 2: 10 pmol/L RSV.5/DR1*0701 template and 0.5 μmol/L primers 1 and 2; same as PCR1, but annealing temperature was 62.3°C. Reaction 3: 10 pmol/L of product from reactions 1 and 2 were mixed with primers 1 and 4 and incubated at 95°C for 2 minutes followed by five rounds of 95°C for 30 seconds followed by 60.2°C for 30 seconds followed by 72°C for 1 minute. Then five rounds more of the same reaction with annealing temperature at 62.3°C followed by 23 rounds of the same reaction at 64.8°C followed by 72°C for 10 minutes. All reactions used 2 units of PFU turbo polymerase (Invitrogen) according to the manufacturer's specifications. These primers added the *Xma*I and *Not*I restriction sites on the 5′ and 3′ ends of the cDNA, respectively. The corrected sequence was confirmed by sequencing of both strands.

Using the same restriction sites as for cloning of pLNCX2/HLA-DR1, the *HLA-DRB1*0701* or *HLA-DRB1*0401* genes were cloned into the downstream site of the pIRES/DRA0101 vector containing the *HLA-DRA*0101* gene in the upstream site. The DRA0101-IRES-DRB1 section was excised from the resulting vector and cloned into the retroviral vector pLNCX2(*AvrII*). The pLHCX/CD80 retroviral construct, retrovirus production, and transductions were previously described (20).

Transduced cells were selected as follows: CD80 transductants (150 μg/mL hygromycin); DR4, DR7, and CIITA transductants (300 μg/mL G418); siRNA transductants (0.3 μg/mL puromycin). If 2 to 3 weeks of drug selection did not yield homogeneous populations of transgene-expressing cells, the transductants were sorted by magnetic bead selection (Miltenyi, Auburn, CA) according to the manufacturer's directions.

Peptides, antibodies, reagents, and immunofluorescence. HER-2/*neu* peptide 98-114 (RLRIVRGTQLFEDNYAL) and peptide 776-790 (GVGSPYVSRLLGICL; refs. 33, 34) were synthesized at the University of Maryland Biopolymer Laboratory. Monoclonal antibodies (mAb: HLA-DR-FITC and CD80-PE), streptavidin-PE, FITC-isotype, and PE-isotype controls were from BD PharMingen (San Diego, CA). Biotinylated HLA-DR1 mAb (BIH0126) was from One Lambda, Inc. (Canoga Park, CA); HLA-DQ-PE and HLA-DP-FITC were from Chemicon (Temecula, CA); rat anti-mouse IgG-FITC was from ICN (Costa Mesa, CA); c-*neu* (Ab-2) was from Oncogene (Cambridge MA); CD4-FITC, CD8-FITC, and anti-human IgG-FITC were from Miltenyi Biotech; and human IgG-FITC was from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A,B,C), L243 (pan anti-HLA-DR), PIN1.1 (anti-Ii), and 28.14.8 (anti-H-$2D^bL^d$) were prepared, and tumor cells and PBMCs were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8, and immunoglobulin) or fixed and stained for Ii as described (20).

Western blots. Ii Western blots were done as described (20). Blots for MHC II were done as for Ii with the following modifications: cell lysates were loaded onto SDS-PAGE gels using nonreducing loading dye [0.2% SDS, 20% glycerol, 1.25 mol/L Tris (pH 6.8), and 0.4 mg/mL bromophenol blue]. Half of each sample was boiled for 5 minutes immediately before loading. Blocking buffer was 2% bovine serum in TBST. Membranes were incubated with undiluted supernatant from hybridoma L243, and the last wash was done for 1 hour.

T-cell priming. PBMCs from healthy donors (2×10^7/4 mL/well) were cultured in PBMC medium [Iscove's modified Dulbecco's medium, 10% FCS, 1% penicillin, 1% streptomycin (BioSource, Rockville, MD), 2 mmol/L Glutamax (Bethesda Research Laboratories/Life Sciences, Grand Island, NY)] with 2 μg/mL of HER2 p98 or p776 in six-well tissue culture plates at 37°C and 5% CO_2 for 5 days. Nonadherent cells were harvested, washed twice with PBMC medium, and replated in 24-well plates with 20 units/mL of recombinant human interleukin 2 (IL-2; R&D Systems, Minneapolis, MN) at 1×10^6/2 mL/well. HER2-activated nonadherent cells were harvested 7 days later; live cells were isolated using Histopaque-1077, cultured 1 to 5 days without exogenous IL-2, and used the following day. For some experiments, after incubation with IL-2, nonadherent PBMCs were cultured at 1×10^7/4 mL/well with 8×10^6, 50 Gy-irradiated SUM/DR7/CD80 cells for 5 days, washed and cultured as above with IL-2 for 7 days, and washed and rested for 1 day before use.

Alternatively, PBMCs were obtained from HER-2/*neu*-immunized patients with stage III or IV breast, ovarian, or non–small cell lung cancer participating in a University of Washington Food and Drug Administration–approved phase I trial (35). Patients were immunized intradermally once a month for 6 months to the same regional draining lymph node site with three different peptides derived from HER-2/*neu* and admixed with 100 μg granulocyte macrophage colony-stimulating factor. PBMCs were

collected 1 month after the last immunization and cryoperserved. For *ex vivo* boost, PBMCs were thawed at 37°C, washed twice, and resuspended at 3×10^6/mL in X-VIVO media [10% human AB serum, 2 mmol/L L-glutamine, 20 mmol/L HEPES buffer, and 10 mmol/L acetylcysteine solution (USP)]. The cells were stimulated with 10 mg/mL of HER-2/*neu* peptides (p98, p776, or p98+p776) and incubated at 37°C in 5% CO_2 for 12 days. On days 4/5 and 8, 10 units/mL of recombinant human IL-2 (Chiron Corp., Emeryville, CA) and 10 ng/mL of recombinant human IL-12 (R&D System) were added. On day 12, the cells were harvested, washed, counted, tested by flow cytometry and enzyme-linked immunospot (ELISPOT), and resuspended at 1×10^6/mL in fresh media containing 1×10^5/mL of anti-CD3/CD28–coated beads to a final concentration of 1 or 10 bead(s) per T cell. Between days 14 and 23, the cell concentration was evaluated every 2 to 3 days, and the cells were diluted to 0.5 to 1×10^6/mL with fresh media as needed. On days 15, 18, 20, and 22, IL-2 was added to a final concentration of 30 units/mL, and on day 25, the expanded cells were harvested, washed, counted, and evaluated by flow cytometry and ELISPOT (36).

Antigen presentation assays. Antigen presentation assays and T-cell depletions were done as described (20) using Miltenyi human CD4 and CD8 beads with the following modifications: stimulator cells were used at 2.5×10^4 per well. MCF10-derived and MCF10A stimulator cells were not irradiated; all other stimulators were 50 Gy irradiated. Antibody blocking experiments included 20 μg/mL L243 (anti-HLA-DR), W6-32 (anti-class I MHC), or 28.14.8. For exogenous HER2 peptide presentation, assays were as for endogenous antigen presentation, except soluble HER2 peptide p98 or p776 was included at 2 μg/mL.

HLA-DR nomenclature and genotypes. Normal donor PBMCs are A24, A29, B44, B35, DR7, DR11, and DRβ3,4. SUM159PT cells are A2, A24, B5, B15, DR4, and DR13. OMM2.3 cells are A11, A29, B7, and B52. MCF10 and MCF10A cells are A33, B55, B22, DR7, and DR4. HLA genotypes were determined by PCR typing and are referred to by their short-hand form (e.g., HLA-DR7 is DRB1*0701).

Statistical analyses. Means, SDs, and statistical significance as measured by Student's *t* test were calculated using Excel v2002.

Results

siRNA down-regulates Ii. The human breast cancer epithelial cell line MCF10 (27), which does not constitutively express MHC class II or Ii molecules, was transduced with a retrovirus encoding the human *CIITA* gene (Fig. 1*A*) and selected by magnetic bead sorting for MHC class II expression. CIITA-transduced MCF10 (MCF10/CIITA) cells are MHC class II (HLA-DR, DP, and DQ) and Ii positive as shown by immunofluorescence (Fig. 1*B*).

Retroviruses expressing siRNAs for Ii were constructed to down-regulate Ii in MCF10/CIITA cells. Four sequences in the human *Ii* gene, starting with AA and having low GC content, were selected (sequences 4, 38, 16, and 50). Double-stranded RNA molecules of these sequences were prepared and were transiently transfected into 293T cells that had previously been transduced with the CIITA retrovirus. Sequences 4 and 50 but not 38 or 16 down-regulated Ii by 4- to 50-fold (data not shown). To obtain stable transductants, sequence 4 was cloned into the pSIREN vector with two different termination signals giving vectors 4.1 and 4.2, with 4.2 containing additional sequence following the six thymidines. Sequence 50 was not used because it contained a four-thymidine repeat that is a stop of transcription for the U6 polymerase III promoter. Additional sequences starting with AA and having low GC content were selected adjacent to sequence 50 and were inserted into pSIREN (sequences 48, 53, and 54). An additional sequence 32 was also randomly selected and inserted into pSIREN. Sequences were inserted using the forward and reverse primers shown in Fig. 1*C*. Retroviruses containing these six siRNAs were prepared and used to transduce MCF10, MCF10/CIITA, and CD80-expressing cells (MCF10/siRNA, MCF10/CIITA/siRNA, MCF10/CIITA/CD80/siRNA cells). Transduced cells were analyzed by flow cytometry 3 days after transduction. Lines containing siRNAs 32, 53, and 48 showed a marked down-regulation of Ii but not complete loss, whereas cells containing siRNAs 4.1, 4.2, and 54 had minimal down-regulation of Ii (data not shown).

Western analyses for Ii (mAb PIN1.1) were done 3 days after transduction to confirm that Ii expression was down-regulated. The predominant form of Ii in MCF10/CIITA cells is p35, with a smaller amount of p33 (Fig. 2*A*). The p35 isoform, which is translated via an alternative translation initiation site, is normally the less abundant isoform of Ii (37). Others have noted an increase in p35 in tumors (38). The p35 and p33 isoforms are both down-regulated >95% in the siRNAs 53, 48, or 32 transductants, and there is a slight down-regulation in siRNA 4.1. siRNAs 4.2 and 54 do not affect Ii expression. Interestingly, at 3 days after transduction, all of the down-regulated cell lines contain a 23-kDa band that corresponds to an Ii degradation product. To ascertain if p23

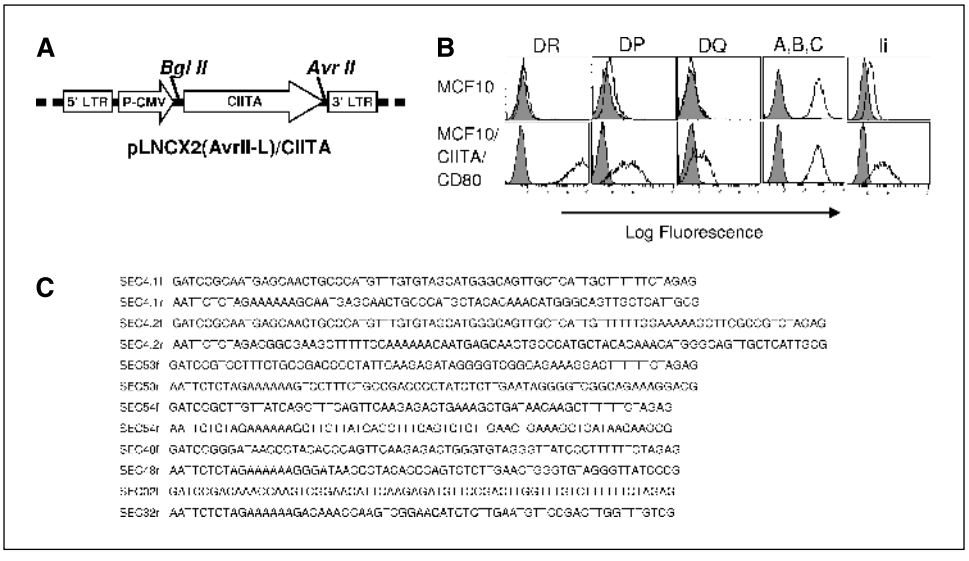

Figure 1. CIITA-transduced breast cancer cells express HLA-DR, HLA-DP, HLA-DQ, HLA-A, HLA-B, HLA-C, and invariant chain. *A,* CIITA retroviral vector. *B,* parental MCF10 and transduced MCF10/CIITA/CD80 cells were stained for HLA-DR (mAb L243), HLA-DQ (mAb CBL118P), HLA-DP (mAb CBL100F), HLA-A,B,C (mAb W6/32), or Ii (PIN1.1) and analyzed by flow cytometry. *Unfilled peaks,* specific antibody-stained cells; *filled peaks,* isotype control stained cells. *C,* forward (*f*) and reverse (*r*) oligonucleotides used to construct siRNA cassettes homologous to human Ii mRNA.

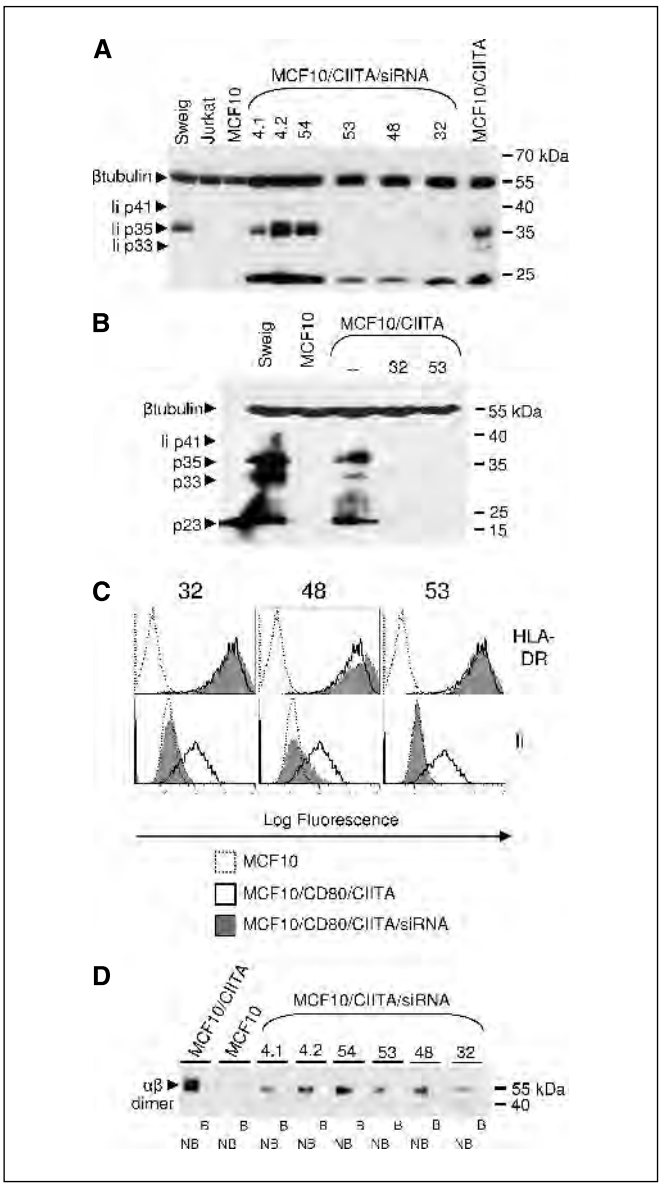

Figure 2. Ii siRNAs silence Ii expression in CIITA-transduced cells without altering HLA-DR expression. MCF10 cells were transduced with retroviruses containing the CIITA and siRNAs for Ii. *A* and *B,* Western blots of MCF10, transduced MCF10, and control Sweig and Jurkat cells probed for Ii with the PIN1.1 mAb, 3 days after transduction (*A*) or 3 weeks after transduction (*B*). *C,* parental and transduced MCF10 cells stained for HLA-DR (mAb L243) or Ii (mAb PIN1.1). *D,* Western blots of parental and transduced MCF10 cells probed for HLA-DR (mAb L243) after 3 weeks of drug selection. Samples were either boiled (*B*) or not boiled (*NB*) before loading on the gel. 4.1, 4.2, 32, 48, 53, 54 identify independent Ii siRNAs.

persists, MCF10/CIITA/siRNA 32 and 53 were cultured in selective media for 3 weeks and subsequently tested by Western analysis. Neither full-length Ii nor the Ii degradation product is present 3 weeks after selection (Fig. 2*B*). Similar results were obtained with MCF10/CD80/CIITA/siRNA cells (data not shown).

To determine if siRNA-mediated down-regulation of Ii affected cell surface expression of MHC class II molecules, the transductants were analyzed by immunofluorescence 3 weeks after siRNA transduction. HLA-DR expression is the same in siRNA and non-siRNA transductants (Fig. 2*C, top*). Ii is absent in MCF10/CD80/CIITA/siRNA 53 and reduced >95% in lines 32 and 48 (*bottom*). Therefore, down-regulation of Ii by siRNA eliminates Ii expression without affecting MHC class II expression.

MCF10/CIITA/CD80 cells down-regulated for Ii contain stable MHC class II heterodimers. Formation of stable αβ heterodimers that dissociate upon boiling is a hallmark of correctly conformed MHC class II molecules (39). To determine if the siRNA transductants have properly conformed MHC II molecules, lysates of >3-week drug-selected cells were either boiled or not boiled before Western blotting with the L243 mAb, which is specific for MHC II αβ dimers. Nonboiled samples contain bands migrating at ~55 kDa that correspond to stable MHC class II αβ heterodimers (Fig. 2*D*). Therefore, tumor cells transduced with the CIITA and Ii siRNA express properly conformed MHC class II molecules in the absence of Ii.

MCF10/CIITA/CD80/siRNA cells present endogenous HER-2/*neu* peptides and activate CD4$^+$ T cells. To assess if MHC class II is functional in the absence of Ii, MCF10/CD80/CIITA/siRNA 32 cells, which are down-regulated for Ii >99%, were used as APCs for the activation of PBMCs to tumor-encoded epitopes. HER-2/*neu*, a growth factor receptor that is overexpressed by many tumors, was used as the tumor antigen because (*a*) MCF10 cells constitutively overexpress HER-2/*neu* (Fig. 3*A*); (*b*) HER-2/*neu* contains two HLA-DR7-restricted peptides (p98 and p776; refs. 33, 34); and (*c*) MCF10/CD80/CIITA cells express DR7. To control for HLA-DR specificity, ocular melanoma OMM2.3 and breast cancer SUM159PT were transduced with CD80 and DR7 or DR4 retroviruses (Fig. 3*B*). OMM2.3 and SUM159PT cells express HER-2/*neu* and do not express Ii, and the respective transductants express CD80 and DR4 or DR7 (Fig. 3*C*).

To determine if the CIITA/siRNA cells present endogenously synthesized tumor peptides, HER-2/*neu* peptide p98 and/or p776-activated T cells were cocultured with transduced tumor cells, and IFN-γ production was measured. Soluble peptide p98 or p776 was added to some wells to determine if the transductants preferentially present peptides from exogenous sources. MCF10/CIITA/CD80/siRNA 32 and MCF10/CIITA/CD80 present peptides p98 and p776 from endogenously synthesized HER-2/*neu* (Fig. 4*A* and *B*). Peptide p776 is presented equally well by MCF10/CIITA/CD80/siRNA 32 and MCF10/CIITA/CD80, whereas MCF10/CIITA/CD80 cells are slightly better presenters of p98 ($P < 0.05$). Both MCF10 cell lines present exogenously pulsed HER-2/*neu* peptides. Exogenous peptide presentation is not significantly better than presentation of endogenous peptide and is not affected by Ii expression. HER-2/*neu* presentation is DR7 restricted because cells lacking DR7 (MCF10/CD80, OMM2.3/DR4/CD80, and SUM159/CD80) do not induce significant IFN-γ release. T-cell activation is limited to MHC-restricted antigen, and there is no activation to allogeneic MHC antigens because neither MCF10/CD80 nor SUM159/CD80 cells induce significant IFN-γ release. Therefore, down-regulation of Ii by siRNA slightly diminishes presentation of peptide p98 but does not significantly affect presentation of p776, suggesting that presentation of some epitopes may be Ii dependent, whereas presentation of others is independent of Ii. In either case, removal of Ii does not render MHC class II molecules unable to present antigen, indicating that MHC class II antigens are functional in the absence of Ii.

To rule out that PBMCs were responding to secreted HER-2/*neu* that is subsequently endocytosed, PBMCs were mixed with supernatants from MCF10/CIITA/CD80/siRNA 32 cells. PBMC were pulsed with p98 as a positive control. Peptide-pulsed PBMC

produced IFN-γ; however, supernatant-pulsed cells did not (data not shown). Therefore, MCF10/CIITA/CD80/siRNA 32 cells are presenting endogenously synthesized molecules.

Peptides p98 and p776 also activate $CD8^+$ T cells, suggesting that they may contain nested MHC class I epitopes (33, 34). The HER-2/*neu*-activated T cells share DR7 with the transduced MCF10 cells, and DR7 and A24 with SUM159PT/DR7/CD80 cells. No MHC class I alleles are common between p98- and p776-activated PBMCs and transduced MCF10 cells. Because peptides p98 and p776 are presented by both DR4 and DR7, there is the potential for the activation of both $CD4^+$ and $CD8^+$ T cells by SUM159PT/DR7/CD80 but not by MCF10/CIITA/CD80/siRNA 32 cells. To identify which T cells are activated, PBMCs were primed with p98 and p776 and subsequently incubated with vaccine cells in the presence of antibodies to MHC class I and/or MHC class II. Antibodies to MHC class II block antigen presentation by MCF10/CIITA/CD80/siRNA 32 and MCF/CIITA/CD80, whereas antibodies to both MHC I and II block antigen presentation by SUM159/DR7/CD80 cells (Fig. 4*C*). To confirm the activation of $CD4^+$ and $CD8^+$ T cells, PBMCs were primed with p776 and depleted for $CD4^+$ or $CD8^+$ T cells, before activation by MCF10 or SUM159PT transductants. Depletion of $CD4^+$ T cells completely eliminates T-cell activation by both MCF10/CIITA/CD80/32 and SUM/DR7/CD80 cells. Depletion of $CD8^+$ T cells significantly reduces T-cell activation by SUM159PT transductants and has a smaller effect on MCF10/CIITA/CD80/32-induced T-cell activation (Fig. 4*D* and *E*). This latter effect is probably nonspecific because MCF10/CIITA/CD80/32 cells do not share MHC class I alleles with PBMCs. Similar results were obtained with p98-primed PBMCs (data not shown).

MCF10/CIITA/32 cells were also included in this experiment (Fig. 4*D*) to determine if coexpression of CD80 enhances boosting of HER-2/*neu*-specific $CD4^+$ T cells. In agreement with earlier findings (20), CD80-expressing transductants are better stimulators. Therefore, the transductants activate both $CD4^+$ and $CD8^+$ T cells if they share common alleles with the responding PBMCs, and coexpression of CD80 enhances activation.

Nonmalignant cells do not activate T cells. A potential problem with cell-based vaccines is that they will activate T cells against nonmalignant cells due to cross-reactivity with normal self-antigens. To determine if the MHC II vaccines induce reactivity against nonmalignant cells, PBMCs were activated with HER-2/*neu* p776 peptide and tested on MCF10 cells and their nonmalignant counterpart, MCF10A cells. As measured by flow cytometry, MCF10A cells express HER-2/*neu*, although at slightly lower levels than MCF10 cells (Fig. 5*A* compare with Fig. 3*A*). Unlike MCF10 cells, MCF10A cells do not express MHC II molecules; however, they are inducible for MHC II if incubated for 48 hours with 1,000 units/mL of rIFNγ. As seen in Fig. 5*B*, neither untreated nor IFNγ-treated MCF10A cells activate T cells. Therefore, tumor-specific T cells are activated by MHC II^+CD80^+ tumor cell–based vaccines and not by nonmalignant cells of the same tissue origin.

Discussion

A goal of tumor immunotherapy is to activate T lymphocytes to tumor-encoded antigens. Although some tumor peptides have been identified, many are unknown, and it is unclear how diverse an immune response is needed to eradicate tumor cells *in vivo*. The

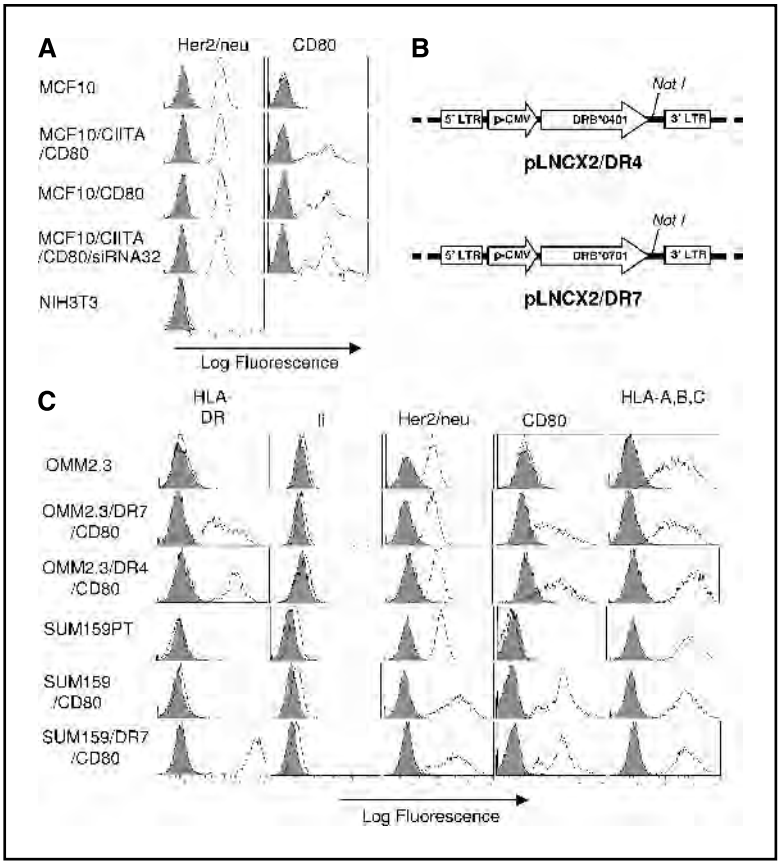

Figure 3. MCF10, OMM2.3, and SUM159PT cells overexpress HER-2/*neu* and transduced HLA genes and do not express Ii. *A,* parental and transduced MCF10 and control NIH3T3 cells labeled with antibodies to HER-2/*neu* or CD80 and analyzed by flow cytometry. *B,* HLA-DR4 and HLA-DR7 retroviral constructs. *C,* parental and transduced OMM2.3 and SUM159 cells stained with antibodies to HLA-DR (mAb L243), Ii (mAb PIN1.1), HER-2/*neu*, CD80, or HLA-A,B,C and analyzed by flow cytometry. *Unfilled peaks,* specific antibody-stained cells; *filled peaks,* isotype control stained cells.

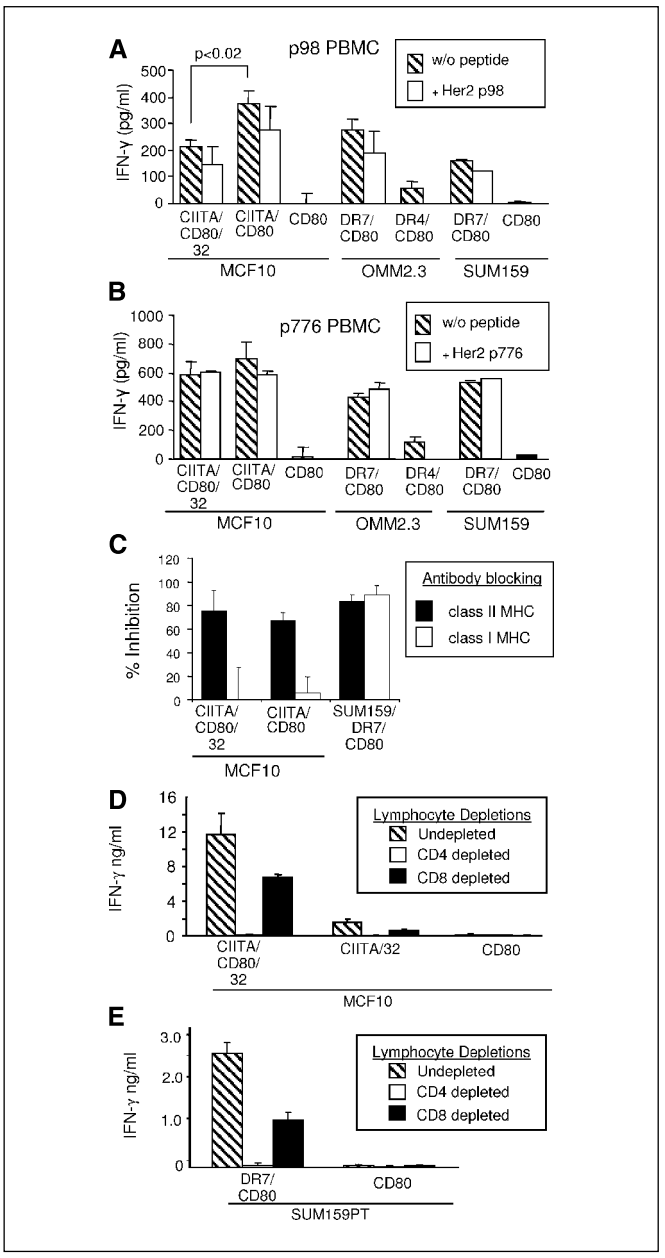

Figure 4. CIITA- and CD80-transduced MCF10 cells down-regulated for Ii by RNAi present endogenously synthesized HER-2/*neu* epitopes and activate $CD4^+$ T cells. HLA-DR7-restricted peptide 98–specific T cells (*A*) or peptide 776–specific T cells (*B*) were cocultured with MCF10, OMM2.3, or SUM159PT parental cells or transductants, and T-cell activation was quantified by measuring IFN-γ release. Exogenous peptide was added to some wells. *C,* p98- and p776-primed T cells were cocultured with transduced MCF10 or SUM159PT cells in the presence of antibodies to HLA-DR (mAb L243) or HLA-A,B,C (mAb W6/32). HLA-DR7-restricted, p776-primed PBMCs were depleted for $CD4^+$ or $CD8^+$ T cells before incubation with MCF10 (*D*) or SUM159PT (*E*) transductants as in (*A*) and (*B*).

vaccines (transductants) described here circumvent the identification of tumor antigens and potentially present a diversity of immunogenic peptides. The vaccines were designed to preferentially present endogenously synthesized MHC II–restricted epitopes (1). In previous studies, we made vaccines by transducing MHC class II alleles into Ii-negative tumor cells (20, 23, 24, 40, 41). We now show that immunogenic cells can also be made by transducing tumor cells with the CIITA with or without Ii.

Our vaccine design was based on the hypothesis that coexpression of Ii would inhibit the presentation of endogenously synthesized tumor peptides, a hypothesis supported by our own earlier work and extensive work of others in nontumor systems (8, 9). Recent mass spectroscopy studies (42) provide direct biochemical support for this hypothesis and also provide an explanation for the lack of inhibition of Ii for HER-2/*neu* peptides p98 and p776. These investigators showed that the MHC II molecules of MHC II^+Ii^- cells contain peptides presented by MHC II^+Ii^+ cells plus additional novel peptides, which are not presented by MHC II^+Ii^+ cells (42). Because p98 and p776 were originally identified as epitopes presented by MHC II^+Ii^+ professional APCs (33, 34), they are most likely in the category of epitopes that are presented by both Ii^+ and Ii^- APCs. However, the findings of ref. (42) make it likely that the MHC II^+Ii^- transductants also present novel tumor antigen epitopes that are not presented by professional APCs. In this fashion, Ii RNAi vaccines may activate a more diverse repertoire of tumor-specific $CD4^+$ T cells than professional APCs and may activate T cells that have not previously been tolerized by the tumor.

Clinical studies also support an inhibitory role for Ii. Chamuleau et al. have shown that acute myelogenous leukemia (AML) patients in complete remission whose HLA-DR^+ myeloid leukemic blasts have low levels of the MHC class II–associated Ii peptide (CLIP), a degradation product of Ii, have a significantly better clinical prognosis than patients whose blasts are DR^+CLIP^+ (43). Similar to AML blasts of progressor patients, DM-deficient mice also have DR^+CLIP^+ APCs, which are inefficient presenters of endogenously synthesized molecules (44). Preferential expression of the Ii p35 isoform is also associated with increased malignancy in chronic lymphocytic leukemia, and this effect has been attributed to reduced presentation of endogenously synthesized

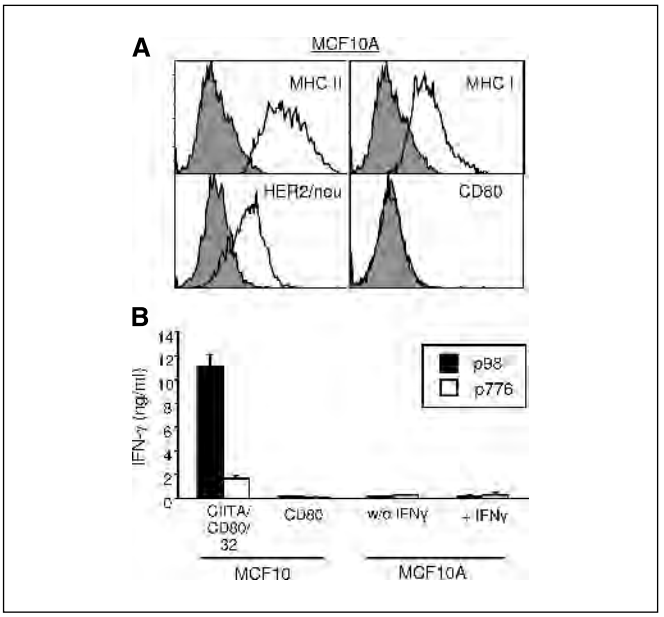

Figure 5. Nonmalignant breast cells do not activate T cells. *A,* untreated MCF10A cells were stained for CD80 or HER-2/*neu*, and IFN-γ-treated (1,000 units for 48 hours) MCF10A cells were stained for HLA-DR or HLA-A,B,C and analyzed by flow cytometry. *Unfilled peaks,* specific antibody-stained cells; *filled peaks,* isotype control stained cells. *B,* PBMCs were pulsed with p776 or p98 and incubated with MCF10, MCF10A, or IFN-γ-treated MCF10A cells, and the supernatants were analyzed for IFN-γ as per Fig. 4.

tumor antigens (38). Expression of CLIP is also associated with polarization towards a type 2 CD4 (Th2) response (45, 46), which may favor tumor progression (47).

Although early studies suggested that Ii expression was essential for MHC class II function (14, 15, 17), there are now many reports showing that MHC II alleles are properly conformed and functional in the absence of Ii (19, 20, 25, 48, 49). The present report extends this conclusion and shows that peptide affinity for MHC II is not affected by Ii, because peptide binding to surface MHC II molecules is similar for Ii^+ and Ii^- cells. Therefore, although Ii may be required during development for expression of some mouse MHC II alleles, most MHC II alleles are stable and functional in the absence of Ii.

We envision that the vaccine strategy described here will be used to generate MHC II allele–specific vaccines from established cell lines. We propose a "cocktail" approach in which a patient will be treated with a mixture of multiple cell lines expressing MHC class I and II molecules matched to their genotype. Following HLA typing, a patient's "semicustomized cocktail" would be prepared from stocks of frozen transduced cells. This approach depends on the existence of shared tumor antigens and eliminates the need for autologous tumor cells, making it feasible to treat most patients. Although retroviruses could be used to induce MHC II and CD80 molecules, alternative techniques that are less controversial would be preferable.

This vaccine strategy has the potential to activate T cells to self-antigens that are also expressed on nonmalignant cells. Autoimmunity has not been observed in the three mouse tumor systems studied *in vivo*,[4] and the absence of reactivity with the nonmalignant breast line MCF10A suggests that autoimmunity against normal cells will also not be a problem in patients. In addition, a $DR4^+DR7^+$ patient with advanced metastatic ocular melanoma has been treated with irradiated OMM2.3/CD80/DR4 and OMM2.3/CD80/DR7 vaccines, and no autoimmune or other complications were noted.[5]

Vaccines consisting of tumor cells transduced with the CIITA and Ii siRNAs have several potential advantages over previous vaccines in which MHC II nonexpressing tumor cells were transduced with individual MHC II alleles. Vaccines made by transducing single *HLA-DR* alleles are limited to presenting tumor antigen epitopes restricted by the transduced allele(s). In contrast, CIITA-transduced vaccine cells express multiple *HLA-DR* alleles, as well as *DP* and *DQ* alleles, and hence have the potential to present a much broader repertoire of tumor antigen epitopes. The CIITA/siRNA vaccines also differ from the previous MHC II vaccines in that expression of the CIITA up-regulates accessory molecules, such as HLA-DM. Although our previous studies have not shown that HLA-DM expression facilitates vaccine efficacy (50), studies by others have shown that HLA-DM expression stabilizes MHC II in the absence of Ii, aids MHC II traffic, and helps edit the MHC II peptide repertoire (42).

The CIITA/Ii siRNA strategy also expands the choice of tumor cells which could be used to generate vaccines to include tumors that constitutively express MHC II or are inducible for MHC II by treatment with IFNγ. Tumor cells that constitutively coexpress MHC II and costimulatory molecules, such as some leukemias (43), are particularly attractive targets for Ii siRNA therapy because creating a vaccine would only require down-regulating Ii via RNA interference (RNAi). This approach is supported by studies in which Ii was down-regulated in MHC II–positive, Ii-positive mouse tumor cells by antisense RNA (51–55). Mice immunized with the Ii antisense down-regulated tumor cells were protected against later challenge with wild-type tumor. Because siRNA is more effective in down-regulating Ii than antisense RNA, Ii siRNA vaccines may have more therapeutic efficacy than Ii antisense vaccines. Therefore, tumor cells expressing the CIITA and costimulatory molecules may be useful reagents, and concomitant down-regulation of Ii via RNAi may further improve vaccine efficacy and protect and/or treat tumor recurrence and/or metastatic disease.

[4] Ostrand-Rosenberg and Clements, unpublished.
[5] S. Slavin, personal communication.

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ORIGINAL ARTICLE

The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4$^+$ T lymphocytes

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Abstract Activation of tumor-reactive T lymphocytes is a promising approach for the prevention and treatment of patients with metastatic cancers. Strategies that activate CD8$^+$ T cells are particularly promising because of the cytotoxicity and specificity of CD8$^+$ T cells for tumor cells. Optimal CD8$^+$ T cell activity requires the co-activation of CD4$^+$ T cells, which are critical for immune memory and protection against latent metastatic disease. Therefore, we are developing "MHC II" vaccines that activate tumor-reactive CD4$^+$ T cells. MHC II vaccines are MHC class I$^+$ tumor cells that are transduced with costimulatory molecules and MHC II alleles syngeneic to the prospective recipient. Because the vaccine cells do not express the MHC II-associated invariant chain (Ii), we hypothesized that they will present *endogenously* synthesized tumor peptides that are not presented by professional Ii$^+$ antigen presenting cells (APC) and will therefore overcome tolerance to activate CD4$^+$ T cells. We now report that MHC II vaccines prepared from human MCF10 mammary carcinoma cells are more efficient than Ii$^+$ APC for priming and boosting Type 1 CD4$^+$ T cells. MHC II vaccines consistently induce greater expansion of CD4$^+$ T cells which secrete more IFNγ and they activate an overlapping, but distinct repertoire of CD4$^+$ T cells as measured by T cell receptor Vβ usage, compared to Ii$^+$ APC. Therefore, the absence of Ii facilitates a robust CD4$^+$ T cell response that includes the presentation of peptides that are presented by traditional APC, as well as peptides that are uniquely presented by the Ii$^-$ vaccine cells.

Keywords Cancer vaccines · Major histocompatibility complex class II · Antigen processing and presentation

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Introduction

Successful cancer vaccines must induce potent tumor-specific immunity and long term immunological memory to protect against recurrent tumors and metastatic disease. Many vaccines are aimed at boosting cell-mediated immunity, which is considered particularly promising due to its ability to destroy tumor cells. Type 1 CD4$^+$ T cells, which secrete IFNγ, have long been recognized as critical components for the activation of CD8$^+$ T cells, either through their classical role as "helper" T cells that provide cytokine support for CD8$^+$ T cells [6, 22], or by their induction of CD40 expression on dendritic cells (DC) ("licensing"), which in turn activate CD8$^+$ T cells [5, 36, 40]. CD4$^+$ T cells are also essential for generating CD8$^+$ T memory cells, for preventing CD8$^+$ T cells from being tolerized [3, 19–21, 41, 44, 45], and for recruiting cells of the innate immune system, such as macrophages. IFNγ production at the tumor site also up-regulates tumor-expressed MHC molecules to improve CTL recognition, and blocks neo-vascularization to prevent tumor proliferation [18, 34, 35]. Because of the central role of CD4$^+$ T cells in facilitating anti-tumor immunity, we are developing vaccine strategies that specifically activate CD4$^+$ T cells.

Activation of CD4$^+$ T cells requires two signals: an antigen-specific signal delivered by a major histocompatibility

complex class II (MHC II) molecule presenting a specific peptide, and a costimulatory signal. Expression of MHC II and costimulatory molecules is usually limited to professional antigen presenting cells (APC), such as DC. Therefore, activation of tumor-reactive $CD4^+$ T cells requires that tumor antigens are endocytosed by DC and processed and presented to the appropriate $CD4^+$ T cells [37]. Presentation of exogenously acquired antigen by DC is facilitated by the expression of the MHC II accessory molecule invariant chain (Ii). As newly synthesized MHC II molecules enter the endoplasmic reticulum (ER), their peptide-binding groove is occupied by Ii. From the ER, MHC II/Ii complexes traffic to endosomal compartments where Ii is degraded, and peptides derived from endocytosed, exogenously synthesized molecules are loaded with the help of HLA-DM [10]. As a result, $CD4^+$ T cells that are activated by professional APC react with antigens obtained from third party (e.g. tumor) cells, but the actual peptides for which the $CD4^+$ T cells are specific, are generated by DC.

Tumors synthesize antigens that are immunogenic and under some circumstances can activate an immune response that mediates tumor rejection [16, 38]. However, many tumor-bearing individuals are tolerized to the tumor-derived peptides presented by their DC, and do not produce tumor-reactive $CD4^+$ T cells [42, 43].

We are developing cell-based "MHC II vaccines" that are designed to overcome an individual's tolerance to their tumor peptides. The MHC II vaccines are tumor cells that are genetically modified to express MHC II and costimulatory molecules. In professional APC which are Ii^+, peptides derived from *exogenously* synthesized molecules are processed in endosomes where they bind to MHC II molecules. Because the vaccine cells do not express Ii, we have hypothesized that MHC II molecules bind peptides in the ER that are derived from *endogenously* synthesized proteins [1, 32, 33]. Since ER-resident peptides are likely to differ from endosomally generated peptides [9], the vaccines may present novel peptides that are not presented by professional Ii positive APC. Therefore, the vaccine cells should activate a different population of $CD4^+$ T cells than that activated by Ii positive APC, thereby overcoming tolerance and stimulating tumor-specific immunity.

In previous reports MHC II vaccines were shown to have significant therapeutic efficacy in mouse models against large, established primary [2] and spontaneously metastatic [30, 31] tumors. We have recently adapted the MHC II vaccine approach for human tumors and have developed several vaccines for human mammary carcinoma and ocular melanoma [13]. Similar to the mouse vaccines, the human MHC II vaccines efficiently present endogenously synthesized tumor peptides, and activate tumor-reactive $CD4^+$ T cells from healthy donors [47] and from patients with primary or metastatic cancer [8]. In this report we demonstrate that human Ii negative MHC II vaccine cells are significantly better activators of tumor-reactive Type 1 $CD4^+$ T cells than are Ii positive APC, and that the Ii negative vaccine cells activate an overlapping, but distinct, repertoire of $CD4^+$ T cells than is activated by Ii positive APC. These findings support our hypothesis that Ii negative MHC II vaccine cells present some tumor peptides that are not presented by professional Ii positive APC and therefore may be effective therapeutic and/or prophylactic agents for cancer patients.

Materials and methods

Cells

The human tumor cell lines Sweig and Jurkat, peripheral blood mononuclear cells (PBMC) from healthy human donors [13], MCF10CA1 human mammary carcinoma (hereafter called MCF10), and MCF10 transductants [47] were handled as described. MCF10/CIITA/CD80/Ii siRNA cells are MCF10/CIITA/CD80 cells that were rendered Ii negative by transduction with Ii siRNA 32. MHC II and Ii siRNA transductants were previously shown to express stable MHC II $\alpha\beta$ heterodimers and not express Ii as assayed by western blotting and flow cytometry [47]. PBMC were obtained by venipuncture from healthy human donors and buffy coats were stored in liquid nitrogen until used. Thawed PBMC were >90% viable. All cell lines and procedures were approved by the institutional review boards of the participating institutions.

Retroviral constructs, transductions and drug selection

The human major histocompatibility complex class II associated invariant chain (Ii) cDNA (Genbank accession no. NID X00497), containing both start codons for p33 and p35 isoforms of Ii, was cloned from sp64/p33 (kindly provided by Eric Long, NIH) [23], into the pLPCX vector (Clontech) containing a puromycin resistance gene. To construct the pLPCX/Ii vector containing the Ii gene, Ii was excised from sp64/p33 with *Sal*I and *Eco*RI (New England Biolabs; Beverly, MA, USA) and inserted into the pLPCX vector digested with *Xho*I (New England Biolabs) and *Eco*RI (*Xho*I having compatible cohesive ends with *Sal*I). Proper insertion of the Ii gene was confirmed by sequence analysis. Sequencing reactions were carried out using the "ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1" (Perkin Elmer Applied Biosystems Division, Foster City, CA, USA) according to the manufacturer's protocol using a PTC-200 Thermal Cycler (MJ Research, BioRad, Hercules, CA, USA) and an ABI PRISM 3700 DNA Analyzer (Applied Biosystem). Retroviruses were prepared, and tumor cells were transduced with the

retroviruses as described [13]. Transduced cells were selected in 0.3 μg/ml puromycin for 2–3 weeks after transduction, and thereafter maintained in MCF10 medium without puromycin. Transduced cells were periodically monitored by flow cytometry for expression of the transduced genes, which were stably expressed for >12 months.

Antibodies, immunofluorescence, western blots

HLA-DR-FITC, CD80–PE, FITC-isotype and PE-isotype controls were from BD Biosciences. CD4-FITC and CD8-FITC were from Miltenyi Biotec Inc. (Auburn, CA, USA); HLA-DM-PE was from BDBiosciences (San Diego, CA, USA). Culture supernatants of hybridomas W6/32 (pan HLA-A, B, C), L243 (pan anti-HLA-DR), and PIN1.1 (anti-Ii) were prepared, and tumor cells and PBMC were stained for cell surface markers, or fixed and stained for Ii as described [13]. T cell receptor (TCR) Vβ repertoire of $CD4^+$ T cells was determined by three color flow cytometry analysis using the IO Test Beta Mark TCR Vβ Repertoire kit (Beckman Coulter, San Diego, CA, USA), which consists of monoclonal antibodies (mAbs) to 24 distinct TCR Vβ families (about 70% coverage of normal human TCR Vβ repertoire). Each test includes three directly coupled TCR Vβ mAbs, conjugated to PE, FITC or both PE and FITC. PBMC were stained and analyzed for TCR Vβ usage according to the manufacturer's instructions, with the following modification: prior to staining with Vβ mAbs, 1×10^5 PBMC were stained with 1 μg of biotin conjugated anti-human CD4 (eBiosciense, SanDiego, CA, USA), then washed and stained with streptavidin-PerCP (BD). Viable $CD4^+$ T cells were gated according to CD4 staining and side scatter, and this population was analyzed for Vβ TCR usage. Western blots were done as described [47].

T cell priming and T cell depletions

Transduced MCF10, (MCF10/DR7/CD80, MCF10/DR7/CD80/Ii, MCF10/CIITA/CD80, and MCF10/CIITA/CD80/Ii siRNA) cells were plated on day 0 in MCF10 medium at 2–4 $\times 10^5$ cells/4 ml in 6 cm dishes and the cells allowed to attach before irradiating with 25 Gy. The next morning MCF10 medium was removed and 1–2 $\times 10^7$ PBMC in 4 ml of T cell medium (Isocoves Modified Dulbeccos Medium, 1% gentamicin, 1% penicillin, 2 mM Glutamax, 0.01 M HEPES, 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), 0.05 mM mercaptoethanol (VWR West Chester, PA, USA), 5% human AB serum (Genini Bio-Products Woodland, CA, USA) were added to each 6 cm dish, and the cells were incubated at 37°C for 3–5 days. At the end of this incubation period, non-adherent cells (PBMC) were harvested, washed twice, and resuspended at 1×10^6 PBMC/2 ml T cell medium supplemented with human recombinant IL-15 (40 ng/2 ml) (Peprotech, Rocky Hill, NJ, USA). PBMC were then plated in 24 well plates at 1×10^6 cells/2 ml/well. After a 7 day culture period, the non-adherent cells ("primed PBMC") were removed, washed twice, and then rested in T cell medium for 1–3 days before being used in antigen presentation assays. In some experiments, PBMC were depleted for $CD4^+$ or $CD8^+$ T cells before priming, using LD columns and CD4 or CD8 microbeads according to the manufacturers' directions (Miltenyi, Auburn, CA, USA).

Antigen presentation assays

MHC II vaccine cells were plated at 2.5×10^4 cells/200μ MCF10 medium/well in 96 well plates and allowed to adhere for 2–4 h. MCF10 medium was then removed and primed PBMC in T cell medium were added at 5×10^4 PBMC/200 μl/well. After 48 h of incubation, the supernatants were analyzed for IFNγ by ELISA [13], and the non-adherent cells were analyzed for TcR Vβ repertoire by flow cytometry. Multiplex cytokine analyses were done in the Cytokine Core Facility at the University of Maryland, Baltimore using the Luminex 100 System (Luminex, Austin, TX, USA) according to the manufacturer's directions.

HLA haplotyping

PBMC from healthy donors and MCF10 cell lines were HLA typed using SSP-ABDR DNA typing trays (OneLambda, Canoga Park, CA, USA) according to the manufacturer's recommendations. MCF10 are A33, B55, DR7. PBMC 100704 are A11, A29; B44, B51; DR4, DR7, DR53. PBMC 111504 are A1, A68; B35, B57; DR1, DR7, DR53; PBMC 123104 are A33, A36; B-, B44; DR1, DR7, DR53.

Statistical analysis and TCR Vβ usage calculations

Mean, SD, and statistical significance as measured by Student's t test were calculated using Excel v2002. Percent increase in TCR Vβ usage = 100% × [(% cells with specific TCR Vβ after activation with Ii^- or Ii^+ APC)/(% cells with specific TCR Vβ before activation)].

Results

MCF10 human mammary carcinoma cells transduced with an Ii (p33) expression vector express Ii

To study the effects of Ii on the repertoire of peptides presented by MHC II-transduced human breast cancer cells, we have constructed an expression vector that contains p33, which is the dominant isoform of Ii [23] (Fig. 1a). MCF10

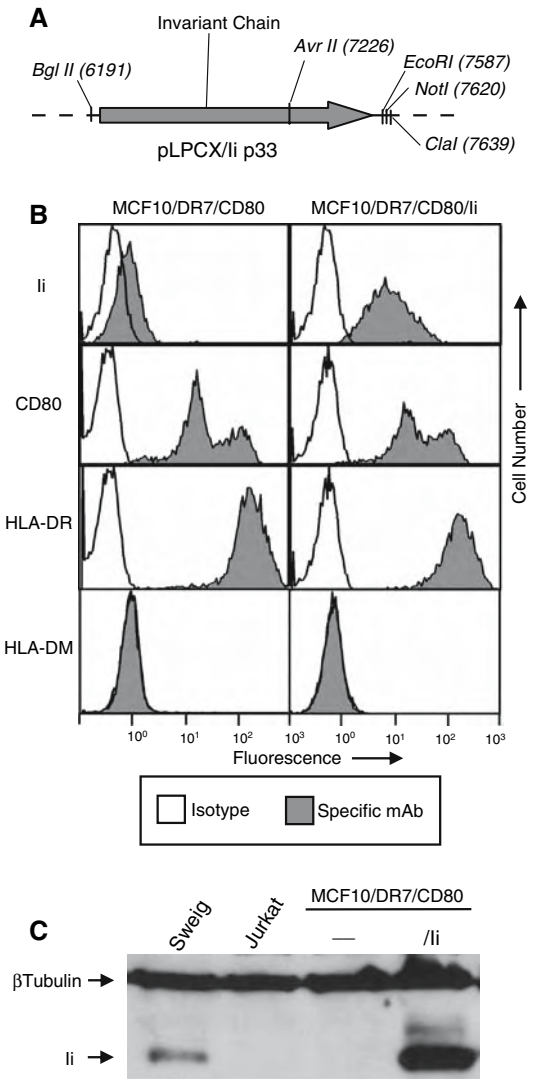

Fig. 1 MHC II breast cancer vaccines prepared from MCF10 cells express HLA-DR and CD80 and do not express invariant chain unless transduced with a p33 Ii construct. **a** Invariant chain retroviral vector. **b** MCF10/DR7/CD80 and MCF10/DR7/CD80/Ii transductants stained for Ii (mAb Pin1.1), CD80 (mAb CD80), HLA-DR (mAb L243), or HLA-DM (mAb DM), and analyzed by flow cytometry. **c** Western blot of MCF10/DR7/CD80 and MCF10/DR7/CD80/Ii cells and control Sweig and Jurkat cells probed for Ii with the PIN1.1 mAb. Data of panels **b** and **c** are representative of three independent experiments

cells previously transduced with retroviruses encoding MHC II and CD80 molecules (MCF10/DR7/CD80) were further transduced with Ii retroviruses. The resulting cells (MCF10/DR7/CD80/Ii) were stained with mAbs for cell surface expression of HLA-DR, HLA-DM, and CD80 (live cells), or for internal Ii expression (fixed cells), and analyzed by flow cytometry (Fig. 1b). MCF10/DR7/CD80/Ii cells express Ii protein, and their levels of HLA-DR and CD80 are similar to the levels expressed by Ii$^-$ MCF10/DR7/CD80 cells. Neither population expresses HLA-DM. Expression of the Ii p33 isoform by MCF10/DR7/CD80/Ii cells was further confirmed by western blotting using the human B cell lymphoma cell line Sweig as a positive control for p33 (Fig. 1c). Therefore, Ii is expressed in MCF10/DR7/CD80/Ii breast cancer cells, and its expression does not alter expression of HLA-DR7, HLA-DM, or CD80.

Ii$^-$ transductants prime CD4$^+$ T cells more efficiently than Ii$^+$ transductants

Previous studies have established that Ii$^-$ MCF10/DR7/CD80 transductants bind MHC II-restricted peptides and activate CD4$^+$ T cells that were primed by peptide pulsing; however, these studies did not determine if the Ii$^-$ vaccines prime CD4$^+$ T cells [47]. To test whether Ii$^-$ vaccine cells efficiently prime CD4$^+$ T cells, HLA-DR7$^+$ PBMC from healthy human donors were depleted for CD8$^+$ T cells and co-cultured (primed) with irradiated MCF10/DR7/CD80 vaccine cells. Following 3 days of culture, non-adherent cells (lymphocytes) were removed, expanded in IL-15 for 6 days, re-cultured (boosted) with MCF10/CD80 or MCF10/DR7/CD80 cells, and the supernatants analyzed for IFNγ (Fig. 2a). Boosting with MCF10/DR7/CD80, but not with the MCF10/CD80, cells induces IFNγ release, indicating that the transduced MHC II allele (DR7) is essential for priming of CD4$^+$ T cells and that allogeneic MHC I differences between the vaccine cells and the responding PBMC do not result in IFNγ release. Therefore, the vaccine cells prime naïve CD4$^+$ T cells and the priming is MHC II-dependent.

To determine if Ii$^+$ cells are as effective as the Ii$^-$ vaccines in priming, DR7$^+$ PBMC from healthy donor 100704 were primed with Ii$^-$ (MCF10/DR7/ CD80) or with Ii$^+$ (MCF10/DR7/CD80/Ii) cells, expanded in IL-15, rested, and boosted with the transductants used for priming. PBMC were either not depleted, or depleted for CD4$^+$ or CD8$^+$ T cells prior to priming (Fig. 2b). Ii$^-$ vaccine cells induced more than twice as much IFNγ production as Ii$^+$ cells and the response is virtually eliminated by depletion of CD4$^+$ T cells prior to priming, indicating that CD4$^+$ T cells are the only cells activated to produce IFNγ. Priming and boosting with Ii$^-$ cells also gave 1.5–2 fold more CD4$^+$ T cells compared to priming and boosting with Ii$^+$ cells (data not shown).

To ascertain the type of response induced by the Ii$^-$ vaccine cells, the supernatants from the boosted cultures of Fig. 2b were assayed by multiplex analysis for IL-2, IL-4, IL-10, IL-15, and IFNγ (Fig. 2c). MCF10/DR7/CD80 vaccine cells induce high levels of the type 1 cytokine IFNγ, intermediate levels of IL-2, and very low levels of the type 2 cytokines IL-4 and IL-10. Therefore, MCF10/DR7/CD80 vaccine cells are significantly more efficient than Ii$^+$ cells for priming type 1 tumor-reactive CD4$^+$ T lymphocytes.

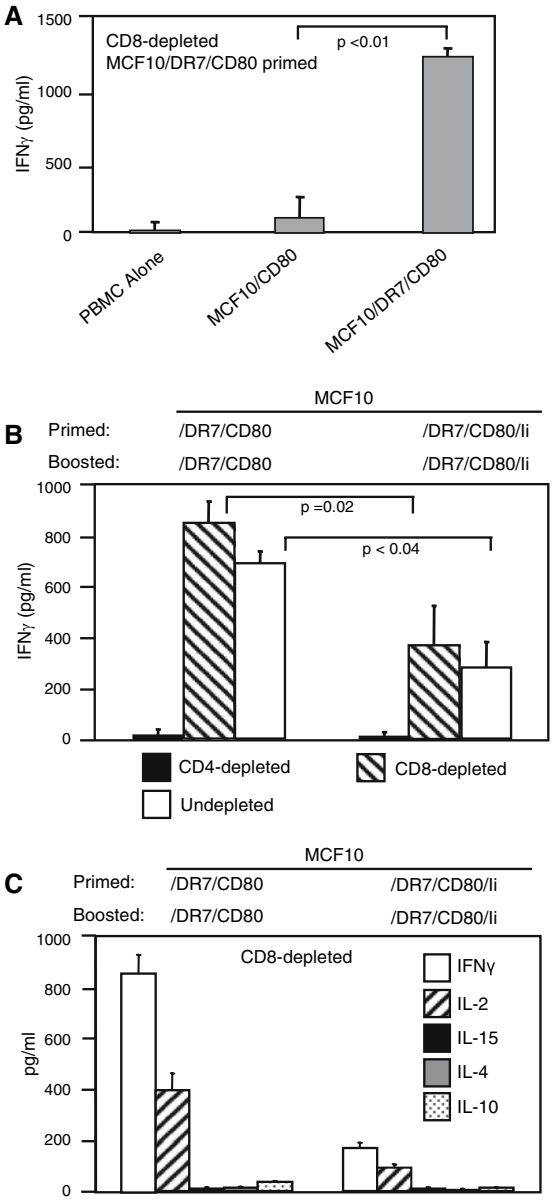

Fig. 2 MCF10/DR7/CD80 vaccine cells prime and boost healthy donor PBMC and are better activators of $CD4^+$ T cells than MCF10/DR7/CD80/Ii cells. **a** PBMC from healthy donor 100704 were depleted for $CD8^+$ T cells, primed with MCF10/DR7/CD80 vaccine cells, boosted with MHC II negative control (MCF10/CD80) or vaccine cells (MCF10/DR7/CD80), and T cell activation quantified by measuring IFNγ release by ELISA. CD8-depleted PBMC contained <2% $CD8^+$ T cells and 88.6% $CD4^+$ T cells at the start of priming. Data are representative of three independent experiments from three different healthy donors. **b** PBMC from healthy donor 100704 were either not depleted or depleted for $CD4^+$ or $CD8^+$ T cells prior to priming and boosting with Ii^- (MCF10/DR7/CD80) or Ii^+ (MCF10/DR7/CD80/Ii) cells. T cell activation was quantified by measuring IFNγ release. After depletion and before priming, CD4-depleted PBMC were <2% $CD4^+$ and 74.3% $CD8^+$; CD8-depleted PBMC were 78.2% $CD4^+$ and <1.5% $CD8^+$; not depleted PBMC were 44.2% $CD4^+$ and 38.3% $CD8^+$. Data are representative of four independent experiments using PBMC from three different healthy donors. **c** Supernatants from primed and boosted PMBC from panel B were tested for multiple cytokines by multiplex analysis

HLA-DM expression does not affect $CD4^+$ T cell activation by MHC II vaccine cells

In professional APC such as DC, HLA-DM facilitates the binding of peptides derived from exogenously synthesized molecules by displacing the class II-associated Ii peptide (CLIP) from the peptide binding groove of MHC II molecules [10]. Since MCF10/DR7/CD80/Ii vaccine cells contain Ii, but do not contain DM (Fig. 1b), their reduced antigen presentation activity may be due to an inability to replace CLIP with other peptides. If MCF10/DR7/CD80/Ii cells have this defect and are unable to replace CLIP, then their cell surface HLA-DR molecules will have bound CLIP. To assess this possibility, live MCF10/DR7/CD80/Ii cells were stained with the CerCLIP mAb which detects MHC II/CLIP complexes (Fig. 3a). MCF10/DR7/CD80/Ii cells express approximately the same amount of HLA-DR/CLIP complexes at the cell surface as the B cell lymphoma Sweig, which constitutively expresses HLA-DR, Ii, and DM. As expected, MCF10/DR7/CD80 cells do not express HLA-DR/CLIP complexes at the cell surface since they do not contain Ii. Therefore, MCF10/DR7/CD80/Ii cells do not express significantly higher levels of CLIP than Sweig cells, making it unlikely that their reduced antigen presentation activity is due to excessive levels of CLIP interfering with MHC II-restricted peptide presentation.

Because professional APC such as DC express DM, we have prepared MHC II vaccines using the MHC class II transactivator (CIITA), which coordinately up-regulates MHC II, Ii, and DM [15], and an siRNA to down-regulate Ii (MCF10/CIITA/CD80/Ii siRNA cells) [47]. As measured by flow cytometry, these vaccine cells express high levels of HLA-DR, very low levels of HLA-DR/CLIP complexes at the cell surface (Fig. 3a), and levels of HLA-DM similar to Sweig cells (Fig. 3b). Since PBMC that are syngeneic to the MCF10 vaccines are not available, and CIITA up-regulates all HLA-DR, -DP, and DQ alleles, the CIITA transductants cannot be used as priming agents because they will induce MHC II allogeneic responses. However, allogeneic T cell activation should not occur during the short duration of the boosting response, so the CIITA transductants can be used as boosting agents. To determine if DM expression impacts the ability of Ii^- vaccine cells to boost $CD4^+$ T cells, $DR7^+$ PBMC from healthy donor 111504 were depleted of $CD8^+$ T cells, primed with Ii^- MCF10/DR7/CD80 cells, and boosted with Ii^-DM^- (MCF10/DR7/CD80), Ii^-DM^+ (MCF10/CIITA/CD80/Ii siRNA), or Ii^+DM^+ (MCF10/CIITA/CD80) cells (Fig. 4a). Boosting with Ii^-DM^- or Ii^-DM^+ cells induces a strong response, while boosting with Ii^+DM^+ cells gives a much poorer response. As expected, allogeneic MHC I differences between the boosting cells and responding PBMC do not activate $CD4^+$ T cells because MCF10/CIITA/CD80 cells do not cause

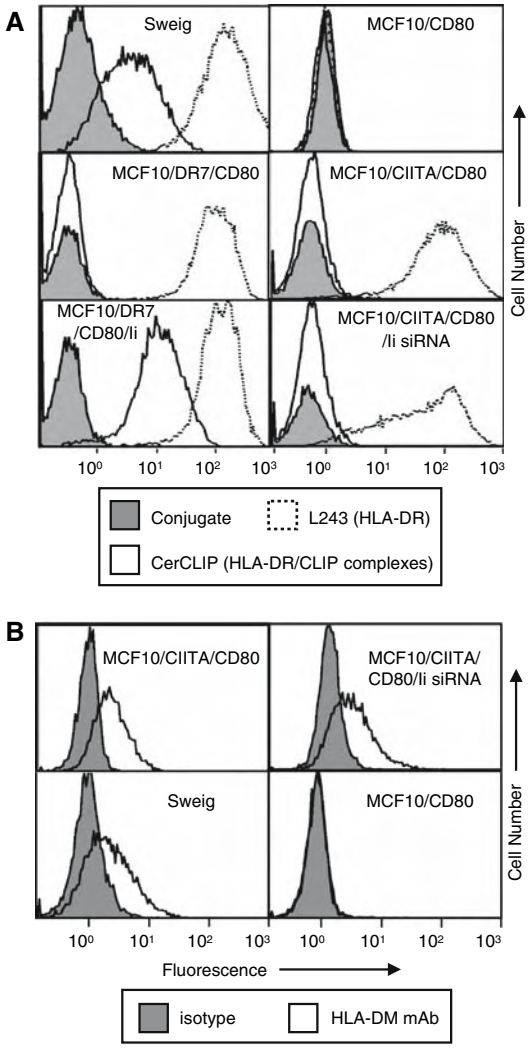

Fig. 3 MHC II vaccine cells do not express HLA-DR/CLIP complexes at the cell surface. Live MCF10 transductants were stained for cell surface HLA-DR/CLIP complexes (CerCLIP mAb), HLA-DR (L243) (**a**), or HLA-DM (**b**) and analyzed by flow cytometry

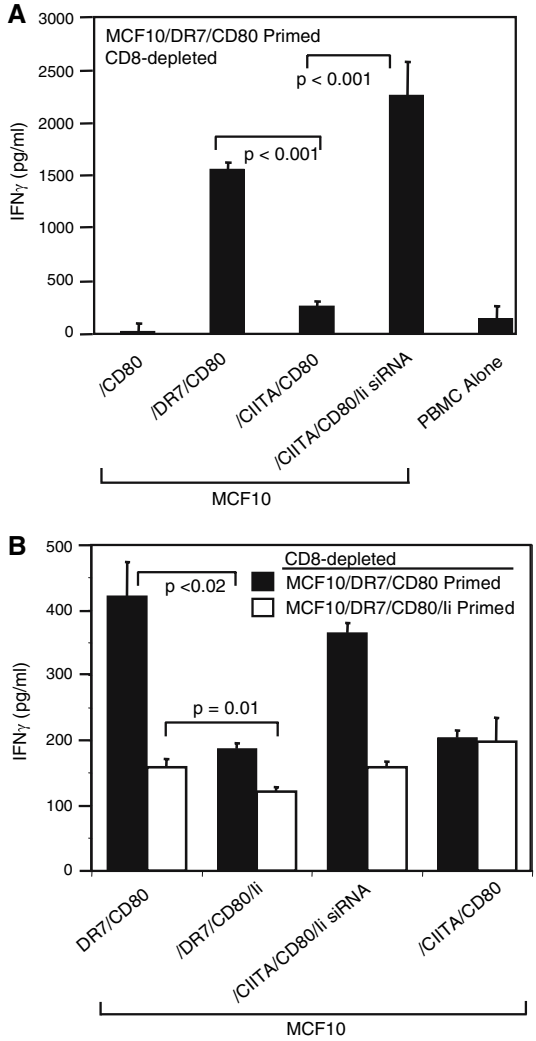

Fig. 4 HLA-DM expression does not affect CD4$^+$ T cell activation by MHC II breast cancer vaccine cells. **a** PBMC from healthy donor 111504 were depleted for CD8$^+$ T cells, primed with Ii$^-$ (MCF10/DR7/CD80) vaccine cells, and boosted with Ii$^-$ (MCF10/DR7/CD80, MCF10/CIITA/CD80/Ii siRNA), Ii$^+$ (MCF10/CIITA/CD80), or control (CD80) transductants. T cell activation was quantified by measuring IFNγ secretion. After depletion and before priming PBMC were 87.5% CD4$^+$ and <2% CD8$^+$. **b** PBMC from DR7$^+$ healthy donor 123104 were depleted for CD8$^+$ T cells, primed with Ii$^-$ (MCF10/DR7/CD80) or Ii$^+$ (MCF10/DR7/CD80/Ii) cells and boosted with Ii$^-$ or Ii$^+$ transductants as in **a**. After depletion and before priming PBMC were 80.7% CD4$^+$ and 4.9% CD8$^+$. Data of panels **a** and **b** are representative of 2–4 independent experiments with 2 healthy donors and 3 experiments with 1 healthy donor, respectively

significant IFNγ release. Therefore, DM expression does not impact the boosting of CD4$^+$ T cells that are primed with Ii$^-$ vaccine cells.

Although DM expression is irrelevant for the boosting of CD4$^+$ T cells that are primed with Ii$^-$ cells, it may affect the boosting of CD4$^+$ T cells that are primed with Ii$^+$ cells. To test this possibility, DR7$^+$ PBMC from healthy donor 123104 were depleted for CD8$^+$ T cells and primed with Ii$^-$ MCF10/DR7/CD80 or Ii$^+$ MCF10/DR7/CD80/Ii cells and boosted with Ii$^-$DM$^-$ (MCF10/DR7/CD80), Ii$^+$DM$^-$ (MCF10/DR7/CD80/Ii), Ii$^-$DM$^+$ (MCF10/CIITA/CD80/Ii siRNA), or Ii$^+$DM$^+$ (MCF10/CIITA/CD80) cells (Fig. 4b). Ii$^-$ cells are significantly more efficient than Ii$^+$ cells for boosting CD4$^+$ T cells, regardless of DM expression. Therefore, Ii$^-$ MHC II vaccine cells are more effective activators of CD4$^+$ T cells than Ii$^+$ cells, and the decreased efficiency of the Ii$^+$ cells is unlinked to HLA-DM expression.

Ii$^-$ and Ii$^+$ vaccine cells activate overlapping, but distinct, repertoires of CD4$^+$ T cells

If Ii$^-$ vaccine cells present novel tumor antigen epitopes that are not presented by Ii$^+$ cells, then Ii$^-$ and Ii$^+$ cells should activate different repertoires of CD4$^+$ T cells. This hypothesis was tested by assessing the TCR Vβ repertoires of the CD4$^+$ T cells activated by Ii$^+$ vs. Ii$^-$ cells. PBMC

from DR7$^+$ healthy human donors were primed and boosted with Ii$^-$ (MCF10/DR7/CD80) or Ii$^+$ (MCF10/DR7/CD80/Ii) cells, and the non-adherent cells were harvested and triple-stained for CD4 and 24 different TcR Vβs. The CD4$^+$ T cells were gated (Fig. 5a) and analyzed for Vβ expression. Figure 5b shows the TCR Vβ family usage by CD4$^+$ T cells from healthy donors 123104 and 100704 before and after activation with either the Ii$^-$ or Ii$^+$ cells. Table 1 shows the number of TCR Vβ families that are increased at least 20% in response to Ii$^-$ or Ii$^+$ cells in these two donors plus a third healthy donor (111504). Some TCR Vβ families are equally activated by both Ii$^-$ and Ii$^+$ cells, while other families are activated exclusively by Ii$^-$ or Ii$^+$ APC. Therefore, Ii$^-$ and Ii$^+$ cells activate some of the same TCR Vβ families, but they also activate distinct families, demonstrating that Ii$^-$ vaccine cells activate CD4$^+$ T cells that are not activated by standard APC that are Ii$^+$.

Table 1 Ii$^-$ and Ii$^+$ breast cancer cells activate over-lapping and distinct repertoires of CD4$^+$ T cells

PBMC donor	Number of TCR Vβ families expanded after activation[a]		
	Ii$^+$ APC	Ii$^-$ APC	Ii$^+$ and Ii$^-$ APC[b]
123104	1	2	6
100704	1	3	10
111504	1	6	5

[a] Expansion of a given TCR Vβ family is defined as a statistically significant increase ($P < 0.5$) of at least 20% in the percent of CD4$^+$ T cells after priming and boosting compared to unprimed CD4$^+$ T cells

[b] Number of common TCR Vβ families expanded by both Ii$^-$ and Ii$^+$ APC

Discussion

The human MHC II vaccines described in this report are generated by transducing Ii$^-$MHC I$^+$ mammary carcinoma cells with CD80 and MHC II alleles syngeneic to the prospective recipient. Previous studies demonstrated that such vaccines induce tumor-specific immunity that results in rejection of established primary and metastatic disease in mouse models [2, 12, 29, 31]. This report extends the mouse studies to humans and demonstrates that the absence of Ii in the vaccine cells is a critical element for vaccine efficacy. We find that MHC II vaccines prime and boost type 1 CD4$^+$ T cells in vitro to release high levels of IFNγ.

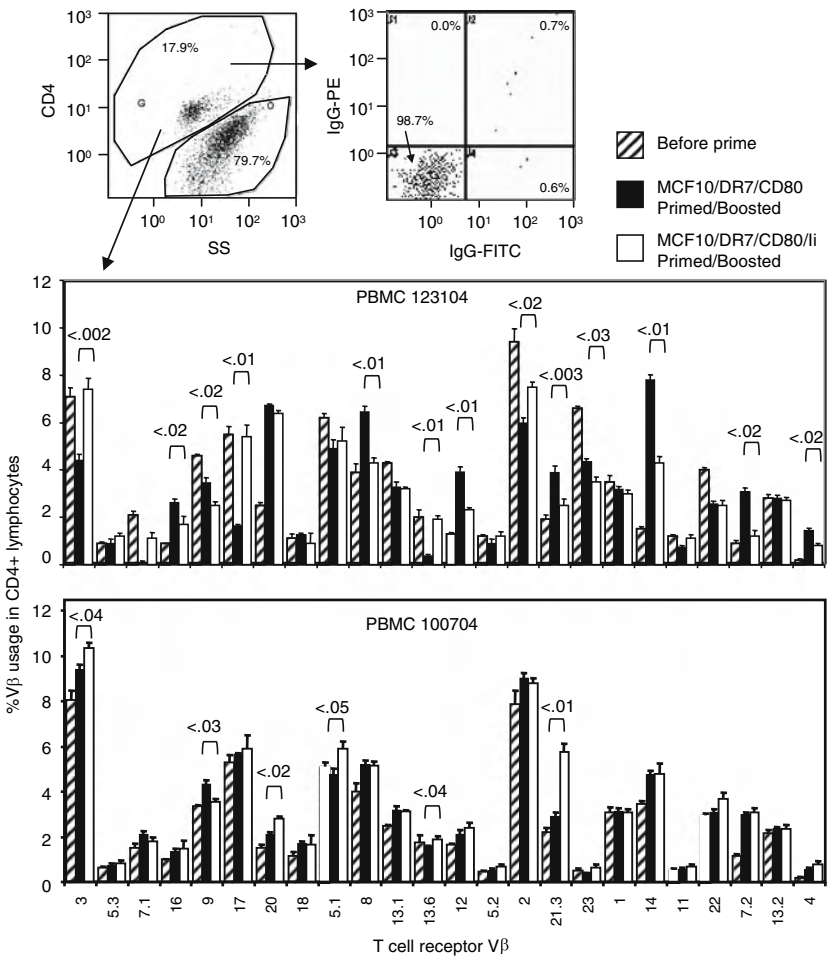

Fig. 5 Ii$^-$ and Ii$^+$ MHC II breast cancer cells activate different repertoires of CD4$^+$ T cells. PBMC from healthy donor 123104 or 100704 were depleted for CD8$^+$ T cells, primed and boosted with Ii$^-$ (MCF10/DR7/CD80) or Ii$^+$ (MCF10/DR7/CD80/Ii) cells and stained with mAb to CD4, mAb to TCR Vβ subtypes, or isotype control mAb. Gated CD4$^+$ T cells were analyzed for the percent of CD4$^+$ T cells expressing a given TCR Vβ. *P* values are indicated. Data are the average of three independent experiments for each PBMC donor

MHC II vaccines consistently induced greater expansion of $CD4^+$ T cells that secreted more IFNγ and expressed a different repertoire of T cell receptor gene families than $CD4^+$ T cells primed and/or boosted by the same cells expressing Ii. Collectively, these observations are consistent with the hypothesis that the absence of Ii facilitates a more robust $CD4^+$ T cell response that includes the presentation of tumor peptides that are presented by Ii^+ APC, as well as distinct peptides that are uniquely presented by Ii^- APC.

There are several possible explanations why the absence of Ii promotes greater expansion of T cells and increased IFNγ secretion by activated $CD4^+$ T cells. In contrast to professional APC, the peptide binding groove of newly synthesized MHC II molecules of MHC II vaccines is not occupied by Ii or CLIP, and may bind peptides derived from endogenously synthesized molecules either in the ER or as they traffic from the ER to endosomes. If the ER contains peptides that are not present in endosomes, then the genetically modified tumor cells may be presenting peptides that are not presented by professional APC. Such antigen presentation would result in the activation of $CD4^+$ T cells that are not typically activated by professional Ii^+ APC. Other studies using different experimental approaches support this possibility. For example, Ii^- MHC II vaccine cells activate $CD4^+$ T cells to peptides derived from diverse subcellular compartments, whereas Ii^+ APC do not [33], consistent with the concept that Ii^- MHC II vaccine cells present a different repertoire of tumor peptides than the repertoire presented by Ii^+ APC. Studies with Ii knockout mice similarly show that the absence of Ii facilitates the presentation of novel endogenous epitopes, while most endogenous antigens that are presented in the presence of Ii are also presented in the absence of Ii [7]. These findings are also corroborated by biochemical studies in which mass spectroscopy analysis of MHC II-bound peptides demonstrated that Ii^- APC display peptides that are not presented by Ii^+ APC [28].

The presentation of different and/or more immunogenic epitopes by Ii^- MHC II vaccines may also be due to the presentation of "cryptic" peptides, a term coined by investigators studying autoimmunity. Cryptic peptides are peptides that are not presented in the thymus during central tolerance induction, but are present in the periphery and which bind with high affinity to MHC molecules. Such peptides have been identified and shown to induce T cell responses to self antigens [4, 17, 24, 39]. If the absence of Ii in MHC II vaccine cells results in altered antigen processing and presentation, as we have hypothesized, then the vaccine cells may present cryptic tumor peptides which potentially induce a more robust $CD4^+$ T cell response.

The MCF10/DR7/CD80 and MCF10/CIITA/CD80/siRNA vaccines both activate tumor-reactive $CD4^+$ T cells in the absence of Ii and have the potential to be clinically useful. We have proposed using the vaccines in non-autologous patients who are HLA-DR-matched to the vaccine cells, to avoid customized vaccine preparation for individual patients [8]. Each vaccine has its pros and cons. Advantages of the multiple allele CIITA-based vaccines include (a) their presentation of tumor peptides on multiple MHC II alleles, (b) their ease of production via a universal construct encoding the CIITA, Ii siRNA, and CD80, (c) the potential to use tumor cells that constitutively express MHC II, and (d) increased expression of MHC class I molecules due to up-regulation of MHC I by the CIITA. A drawback of the CIITA vaccines is their potential to activate allo-MHC II responses, which may overwhelm the tumor-specific MHC II-restricted response in non-fully MHC II-matched recipients. A major advantage of the single allele vaccines is the ease of HLA-DR-matching of the vaccine to an individual patient. A potential disadvantage of the single allele vaccines is that an IFNγ-inducible tumor cell line could be up-regulated for Ii if the vaccine enters or induces an inflammatory environoment. However, this disadvantage can be overcome if non-IFNγ-inducible cells are used or if the cells are co-transduced with Ii siRNA. Both the single allele and multiple allele vaccines could be stored as frozen cells, thereby minimizing reagent preparation time for individual patients.

Several clinical observations support our hypothesis that tumor cell co-expression of Ii with MHC II blocks tumor cell immunogenicity and favors tumor progression by impeding T cell activation to endogenously synthesized tumor antigens. Chamuleau and colleagues have observed that acute myelogenous leukemia patients whose myeloid leukemic blasts are $HLA\text{-}DR^+CLIP^{-\text{ or low}}$ have a significantly better clinical prognosis than patients whose blasts are DR^+CLIP^{high} [11]. Since the failure to remove CLIP from MHC II molecules reduces endogenous antigen presentation [25], this observation is consistent with the concept that HLA-DR expression in the absence of Ii favors the activation of tumor-reactive $CD4^+$ T cells. Similarly, the co-expression of Ii by $HLA\text{-}DR^+$ hepatocellular carcinoma cells is associated with a very poor prognosis [46]. Likewise, tumor cell expression of an isoform of Ii that blocks endogenous antigen presentation is associated with poor prognosis in chronic lymphocytic leukemia patients [48]. If the vaccine cells are the actual APC in vivo, then they may be particularly useful for treating cancer patients whose DC are dysfunctional due to tumor burden [26, 27]. If the vaccine cells are not the relevant APC in vivo, and $CD4^+$ T cells are activated via cross-dressing [14] or by conventional antigen presentation via DC, then the vaccines are useful reagents for producing novel peptide-MHC complexes. Regardless of the precise mechanism by which the MHC II vaccines activate tumor-reactive $CD4^+$ T cells,

they efficiently prime and boost $CD4^+$ T cells, and the absence of Ii is critical for their activity.

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Activation of Tumor-specific CD4$^+$ T Lymphocytes by Major Histocompatibility Complex Class II Tumor Cell Vaccines: A Novel Cell-based Immunotherapy

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ABSTRACT

Mouse tumor cells transfected with syngeneic MHC class II and costimulatory molecule genes are therapeutic vaccines in mice, provided they do not coexpress the class II-associated invariant chain (Ii). We demonstrated previously that the vaccine cells present tumor peptides via the endogenous antigen presentation pathway to activate CD4$^+$ and CD8$^+$ T cells. Because of their efficacy in mice, we are translating this vaccine strategy for clinical use. To obtain MHC class II$^+$CD80$^+$Ii$^-$ human tumor cells, we developed retroviruses encoding HLA-DR and CD80. The HLA-DR virus encodes the DRα and DRβ0101 chains using an internal ribosomal entry site to coordinate expression. SUM159PT mammary carcinoma and Mel 202 ocular melanoma cells transduced with the retroviruses DRB1/CD80 express high levels of DRB0101 and CD80 on the cell surface in the absence of Ii. Irradiated SUM159PT/DR1/CD80 vaccines stimulate proliferation of non-HLA-DRB0101 peripheral blood mononuclear cells and present an exogenous DR1-restricted tetanus toxoid (TT) peptide, indicating that the transduced DRB0101 is functional. SUM159PT/DR1/CD80 vaccines were further transduced with a retrovirus encoding the TT fragment C gene, as a model tumor antigen. These cells stimulate IFN-γ release from TT-primed human DRB0101 peripheral blood mononuclear cells, demonstrating their ability to present "endogenous" tumor antigen. Depletion and antibody blocking experiments confirm that MHC class II-restricted, endogenously synthesized epitopes are presented to CD4$^+$ T cells. Therefore, the MHC class II vaccines are efficient antigen-presenting cells that activate tumor-specific MHC class II-restricted, CD4$^+$ T lymphocytes, and they are a novel and potential immunotherapeutic for metastatic cancers.

INTRODUCTION

A key goal of cancer vaccine development is to generate therapeutic reagents that provide protection against development and outgrowth of metastatic tumor cells. Because metastatic disease for many tumors appears at varied intervals after diagnosis of primary tumor, the most effective vaccines will provide long-term immune memory. We (1, 2) and others (3–5) have focused on the critical role of CD4$^+$ T cells in cancer vaccines, because these cells, in conjunction with CD8$^+$ T lymphocytes, are likely to provide maximal antitumor immunity with long-term immunological memory.

To better activate tumor-specific CD4$^+$ T cells, we have designed cell-based vaccines that facilitate the presentation of MHC class II-restricted tumor peptides to responding CD4$^+$ T cells (2). We have reasoned that tumor cells present a variety of MHC-restricted peptides that are potential tumor antigens, and that if they constitutively express MHC class I molecules and are transduced with syngeneic MHC class II and costimulatory molecules, they could function as antigen-presenting cells (APCs) for MHC class I- and class II-restricted tumor peptides. This approach is appealing for several reasons: (*a*) identification of specific tumor antigen epitopes is not required; (*b*) multiple class I- and class II-restricted epitopes will be presented concurrently; and (*c*) CD4$^+$ T cells may be activated to novel MHC class II-restricted tumor epitopes not presented by professional APCs.

To test our approach, cell-based vaccines were generated from three independent mouse tumors that constitutively express MHC class I molecules and do not express MHC class II molecules (mouse SaI sarcoma, B16 melanoma, and 4T1 mammary carcinoma). The mouse tumor cells were transfected with syngeneic MHC class II α- and β-chain genes and with costimulatory molecule (*CD80*) genes. This vaccine approach was adapted for two reasons:

(*a*) In conventional immunity, activation of CD4$^+$ T cells requires the uptake of soluble antigen by professional APCs and the cross-presentation of the processed antigen to specific CD4$^+$ T cells. If antigen is limiting, as it may be when tumor burden is low, available antigen may not be sufficient for the activation of tumor-specific CD4$^+$ T cells. Our vaccine design bypasses the requirement for professional APCs and soluble tumor antigen because the genetically modified tumor cell vaccines function as the APC.

(*b*) Because each vaccine cell expresses both MHC class I and class II molecules and their associated tumor peptides, a given vaccine cell could be an APC for both MHC class I- and class II-restricted tumor antigen epitopes and concurrently activate both CD4$^+$ and CD8$^+$ T cells. If CD4$^+$ and CD8$^+$ T cells are simultaneously activated by the same APC and are in close proximity to each other, then the transfer of "help" from the activated CD4$^+$ to the CD8$^+$ T cell should be highly efficient (2, 6), thereby maximizing the therapeutic effect. CD4$^+$ T cell "help" could be provided to CD8$^+$ T cells via the classical mechanism of soluble cytokine production or by the alternative mechanism of up-regulation of CD40 on the vaccine cells (APCs; Ref. 7). Regardless of the mechanism of help, the activated CD4$^+$ T cells do not need to directly interact with wild-type tumor cells or with professional APCs, because their only role is to provide help to CD8$^+$ T cells.

Extensive studies using a variety of mouse tumor models have shown that immunization/immunotherapy with the MHC class II plus CD80-modified vaccines induces a potent antitumor immunity against wild-type tumor that confers prophylactic protection (1), delays or eliminates growth of primary solid tumors (8), reduces both experimental (9) and spontaneous metastasis, and extends survival (10, 11). Immunization studies using genetically marked vaccine cells have demonstrated that the vaccine cells themselves are the relevant APCs *in vivo* (12–14), and that both CD4$^+$ and CD8$^+$ T cells are required for the optimal antitumor effect (8, 11). Therefore, by circumventing the traditional cross-presentation pathway for activation of CD4$^+$ and CD8$^+$ T cells, these genetically modified cancer vaccines induce a potent tumor-specific immunity against wild-type tumor cells.

The efficacy of the vaccines depends on their ability to present endogenously synthesized, MHC class II-restricted tumor antigen epitopes to activate CD4$^+$ T cells. Presentation of endogenous antigen

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is favored when levels of the class II-associated accessory molecule, invariant chain (Ii), are limiting (reviewed in Ref. 15). Because MHC class II and Ii are coordinately regulated and Ii expression blocks vaccine efficacy (14, 16, 17), we have used tumor cells that do not constitutively express MHC class II (or Ii) as the "base" line for the vaccines.

Because of its therapeutic efficacy in mice, we are translating this vaccine approach for the treatment of human cancers. Our experimental design is to express syngeneic MHC class II and costimulatory molecules in established human tumor cell lines that constitutively express MHC class I molecules and do not constitutively express MHC class II or Ii. To achieve this goal, we are using retroviral transduction to express HLA-DR and CD80 molecules in two human tumor lines, an ocular melanoma (Mel 202) and a mammary carcinoma (SUM159PT). The resulting HLA-DRB0101-transduced cells stably express high plasma membrane levels of functional HLA-DRB0101, as measured by immunofluorescence, activation of allogeneic peripheral blood mononuclear cells (PBMCs), and presentation of a DR1-restricted peptide. To ascertain that the transductants activate $CD4^+$ T lymphocytes to endogenously synthesized antigens, we have shown that tetanus toxoid (TT) fragment C-transduced vaccine cells activate TT-specific HLA-DRB0101-restricted $CD4^+$ T cells. Therefore, human tumor cells genetically modified by gene transfer to express syngeneic MHC class II and costimulatory molecules express functional HLA-DR molecules and may serve as useful therapeutics for activating tumor-specific $CD4^+$ T lymphocytes of cancer patients.

MATERIALS AND METHODS

Construction of Retroviral Vectors. For the pLNCX2/DR1 construct, DRA cDNA in the RSV.5 vector (18) was PCR amplified including 5′ *Nhe*I and 3′ *Xho*I restriction sites: DRA 5′ primer, TGTCGCTAGCATGGCCATA-AGTGGAGT; and DRA 3′ primer, ACTGCTCGAGTTACAGAGGCCCC-CTGCGTT. The PCR product was cloned into the pCR2.1-TA vector (Invitrogen, Carlsbad, CA), excised with *Nhe*I and *Eco*RI, and inserted into the multiple cloning site (MCS)-A of *Nhe*I- and *Eco*RI-digested pIRES plasmid (Clontech, Palo Alto, CA). DRB0101 in the RSV.5 vector (18) was PCR amplified including 5′ *Xma*I and 3′ *Not*I sites and subcloned into the 5′ *Xma*I and 3′ *Not*I sites of the MCS-B of the pIRES vector: DRB0101 5′ primer, AG-TACCCGGGATGGTGTGTCTGAAGCTC; and DRB01013′ primer, TAG-TGCGGCCGCTCAGCTCAGGAATCCTGTTG. PCR conditions for both DRA and DRB0101 amplifications were: denature at 94°C for 2 min, denature at 94°C for 1 min, anneal at 60.9°C or 62.9°C (DRA and DRB0101, respectively) for 1 min, extend at 72°C for 3 min (High Fidelity Taq; Roche, Basel, Switzerland); repeat the last three steps 30 times and extend at 72°C for 7 min. The resulting construct is pIRES/DR1 (Fig. 1*A*).

The pLNCX2 retroviral vector (Clontech) was modified to include a linker containing an *Avr*II site in the MCS. To make the linker, equimolar amounts oftheoligonucleotides(5′-GATCTCGAGCTCCTAGGAATTGTTTGGCCGA-GGC-3′ and 3′-AGCTCGAGGATCCTTAACAAACCGGCTCCGCCGG5-′) were mixed, heated at 95°C for 5 min, and then incubated at 22°C for 1 h. The resulting linker was ligated to *Bgl*II- and *Not*I-digested pLNCX2. The resulting construct is pLNCX2/*Avr*II.

The DRA-IRES-DRB0101 fragment of the pIRES/DR1 was digested with *Nhe*I and *Not*I and gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA) and then ligated to *Avr*II- and *Not*I-digested pLNCX2/*Avr*II. The final MHC class II construct is pLNCX2/DR1 (Fig. 1*A*).

For the pLHCX/CD80(HPH) construct, pLHCX (hygromycin resistance; Clontech) was modified to include a 5′ *Bam*HI site and a 3′ *Hin*dIII site by inserting an oligonucleotide linker between the *Hin*dIII and *Cla*I sites of the MCS. The original *Hin*dIII in the vector was deleted by insertion of the linker. *Xho*I, *Hpa*I, *Avr*II, and *Not*I restriction sites were included in the linker for future cloning purposes. The linker sequence was: L1, 5′-AGCTGCTCGAGT-TAACGGATCCTAGGAAGCTTGCGGCCGCAT-3′; and L2, 5′-CGAT-GCGGCCGCAAGCTTCCTAGGATCCGTTAACTCGAGC-3′.

Human CD80 was excised from the pREP10/B7.1 vector with *Bam*HI and *Hin*dIII and inserted into the modified pLHCX vector using the *Bam*HI and *Hin*dIII sites (Fig. 1*B*).

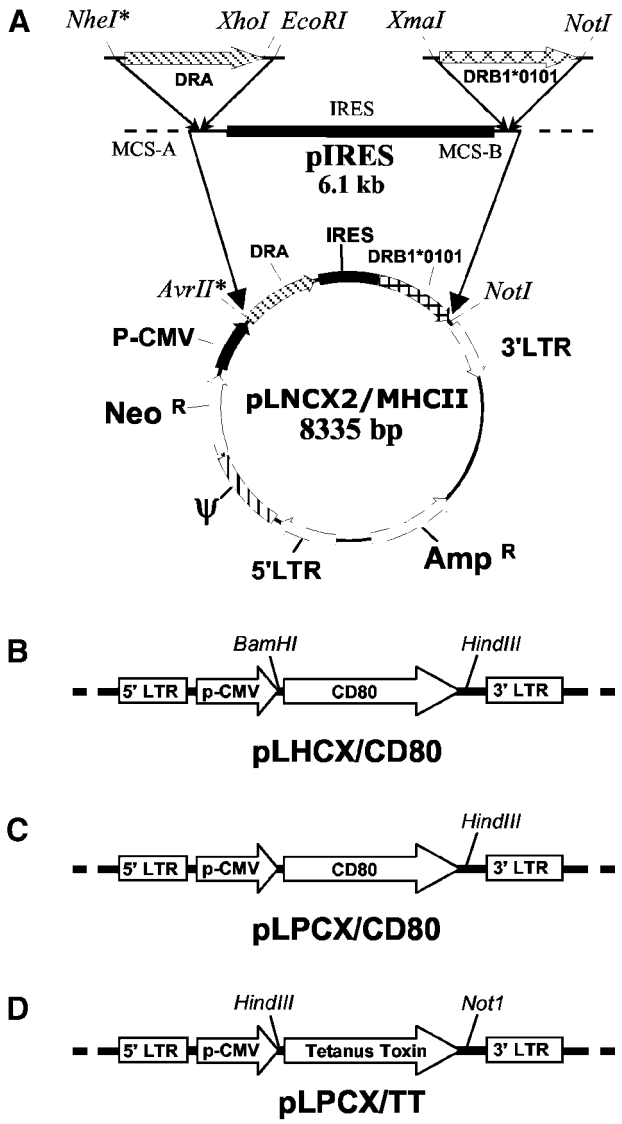

Fig. 1. Retroviral constructs made and used in these studies. *A*, the pLNCX2/MHC II construct contains the DRA and DRB0101 cDNAs flanking an IRES and under the control of the cytomegalovirus (*CMV*) promoter and contains the *G418* resistance gene. *B* and *C*, the pLHCX/CD80 and pLPCX/CD80 constructs encode the human *CD80* gene and contain the hygromycin or puromycin resistance genes, respectively. *D*, the pLPCX/TT construct encodes the TT fragment C gene and contains the puromycin resistance gene. *LTR*, long terminal repeat.

For the pLPCX/CD80 (Puro) construct, the *CD80* gene was excised from pREP10/B7.1 by digestion with *Bgl*II and *Hin*dIII and ligated into pLPCX digested with *Bam*HI and *Hin*dIII. The *Bam*HI and *Bgl*II sites were deleted during this process (Fig. 1*C*).

For the pLPCX/TT construct, TT fragment C DNA was PCR amplified from pCR Blunt (19) to include an ATG start codon and *Hin*dIII site at the 5′ end and a *Bam*HI site at the 3′ end: 5′ primer sequence, CCGCCGAAGCT-TGCCACCATGAAAAACCTTGATTGTT; and 3′ primer sequence, CTGT-TCGGATCCTTAGTCGTTGGTCCAA. PCR conditions were: denature at 94°C for 5 min, denature at 94°C for 1 min, anneal at 55°C for 1 min, extend at 72°C for 1 min (*Taq*DNA polymerase; Invitrogen); repeat the last three steps 35 times and extend at 72°C for 10 min. The resulting PCR product was inserted into the TA cloning vector, pGEM-T-Easy (Invitrogen). The modified TT fragment C gene was then excised with *Hin*dIII and *Bam*HI and inserted into the mammalian expression vector pCDNA3.1/Zeo(+) (Invitrogen). A *Hin*dIII-*Not*I fragment containing the TT fragment C gene was then excised from pCDNA3.1/Zeo(+) and subcloned into the *Hin*dIII-*Not*I site of the MCS of pLPCX(Puro) to produce the pLPCX/TT vector.

Table 1 *Tumor cell vaccines (transductants) used in these studies*

Cell line	HLA-DRB0101	CD80	TT[a]	Drug selection
SUM/DR1	+			G418[b]
SUM/CD80		+		HPH[c]
SUM/TT			+	Puro[d]
SUM/DR1/CD80	+	+		G418[b] + Puro[d]
SUM/DR1/CD80/TT	+	+	+	G418[b] + HPH[c] + Puro[d]
SUM/DR1/TT	+		+	G418[b] + Puro[d]
SUM/CD80/TT		+	+	HPH[c] + Puro[d]
Mel 202/DR1	+			G418[b]
Mel 202/CD80		+		HPH[e]
Mel 202/DR1/CD80	+	+		G418[b] + Puro[d]

[a] Tetanus toxin fragment C.
[b] 600 μg/ml.
[c] 200 μg/ml.
[d] 0.2 μg/ml.
[e] 75 μg/ml.

Cells. Media for all cell lines contained 1% gentamicin, 1% penicillin/streptomycin (all from BioSource, Rockville, MD), and 2 mM Glutamax (BRL/Life Sciences, Grand Island, NY). All cells and T-cell activation assays were cultured at 37°C in 5% CO_2. SUM159PT was obtained from the Michigan Breast Cell/Tissue Bank[3] and was maintained in Ham's F-12 medium with 10% heat-inactivated FCS (Hyclone, Logan, UT), 1 μg/ml hydrocortisone, and 5 μg/ml insulin (both from Sigma, St. Louis, MO). Mel 202 (20) was grown in RPMI 1640 (BioSource, Rockville, MD) with 10% FCS, 0.01 M HEPES (Invitrogen, Grand Island, NY), and 5×10^{-5} M β-mercaptoethanol (J. T. Baker, Inc., Phillipsburg, NJ). Transductants were grown in the same medium as their parental cells, supplemented with G418 (Sigma), puromycin (Clontech, Palo Alto, CA), or hygromycin (Calbiochem, San Diego, CA; see Table 1 for dosages), depending on their transgenes. Sweig and Jurkat cells were obtained from the American Type Culture Collection and were maintained in Iscove's modified Dulbecco's medium (BioSource) supplemented with 10% fetal clone I (FBP; Hyclone). EBV B cells were grown in RPMI 1640 with 10% FCS and 0.01 M β-mercaptoethanol. Peripheral blood mononuclear cells (PBMCs) were grown in Iscove's modified Dulbecco's medium with 5% human AB serum (Gemini Bio-Products, Woodland, CA). All cell lines and procedures with human materials were approved by the Institutional Review Boards of the participating institutions.

Retrovirus Production. 293T cells (obtained from the Harvard Gene Therapy Institute) were plated in a 6-cm dish at 9×10^5 cells/4 ml of 293T medium [DMEM (BioSource, Rockville, MD), 1% gentamicin, 1% penicillin/streptomycin, 1% Glutamax, and 10% heat-inactivated FCS] and cultured at 37°C. Twenty h later, the growth medium was replaced with 4 ml of 37°C Iscove's modified Dulbecco's medium containing 25 mM HEPES (BioSource), 1% Glutamax, and 10% heat-inactivated FCS. Three h later, the 293T cells were transfected with pLNCX2/DR0101, pLHCX/CD80, pLPCX/CD80, or pLPCX/TT plasmids (8 μg) plus pMD.MLV gag.pol (6 μg) and pMD.G (2 μg) using $CaPO_4$ (21). Twelve to 16 h after transfection, medium was replaced with 293T growth medium containing 10 mM HEPES. Virus was collected 48 h later and either used immediately or stored at −80°C.

Retroviral Transduction. Tumor cells were plated in 6-well plates at 1.2-3 $\times 10^5$ cells/3 ml growth medium/well. Approximately 16 h after plating, when cells were in log phase, growth medium was replaced with 500 μl of viral supernatant mixed with 500 μl of 293T medium containing 4 μg/ml polybrene (Sigma) and 10 mM Hepes. Cells were incubated for 5–6 hrs at 37°C, washed twice with excess PBS and maintained in growth medium for 2 days before adding G418, puromycin, and/or hygromycin.

Peptides, Antibodies, Reagents, and Immunofluorescence. TT p2 peptide $TT_{830-844}$ (QYIKANSKFIGITEL; Ref. 22) was synthesized at the University of Maryland Biopolymer Laboratory. Formaldehyde-inactivated TT was purchased from Accurate Chemical and Scientific Corporation (Westbury, NY).

Monoclonal antibodies [mAbs; HLA-DR-FITC, CD80-phycoerythrin (PE), and anti-TT], streptavidin-PE, FITC-isotype, and PE-isotype controls were purchased from BD PharMingen (San Diego, CA). Biotinylated HLA-DR0101 was purchased from One Lambda, Inc. (Canoga Park, CA). Rat anti-mouse IgG-FITC was purchased from ICN (Costa Mesa, CA), and CD4-FITC, CD8-FITC, and anti-human IgG-FITC were purchased from Miltenyi Biotech (Auburn, CA). Human IgG-FITC was purchased from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A,B,C), L243 (pan anti-HLA-DR), 28.14.8 (anti-H-$2L^d$, D^b), and PIN1 (anti-Ii) were purified on protein A or protein G affinity columns as described previously (1). Tumor cells and PBMCs were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8, and immunoglobulin) or fixed and stained for internal markers (Ii, TT) by direct or indirect immunofluorescence as described previously (1, 16). PBMCs were collected by venipuncture from HLA-typed healthy donors and isolated using Histopaque 1077 separation medium as described previously (20). For some experiments, PBMCs were provided by Dr. D. Mann (University of Maryland Baltimore). PBMCs were stored at 1×10^7 cells/ml at −80°C until used.

Western Blots. Western blot analyses were performed as described (14) using 10% SDS-PAGE. Blots were incubated with PIN1.1 mAb (0.003 μg/ml) followed by sheep anti-mouse HRP at a 1:10,000 dilution (Amersham).

Allogeneic T-Cell Activation. Responder PBMCs (1×10^5/well) were cultured in triplicate with 5×10^3 or 1×10^4 irradiated (CS-137 irradiator; Kewaunee Scientific, Statesville, NC) stimulator SUM159PT (50 Gy) or 5×10^5 allogeneic PBMCs (40 Gy) per well in 200 μl/well of culture medium (RPMI, 10% FCS, 1% penicillin/streptomycin, 2 mM Glutamax, and 0.01 M β-mercaptoethanol) in flat-bottomed 96-well microtiter plates (Corning, Inc., Corning, NY). Cells were incubated at 37°C in 5% CO_2 for 6 days and pulsed with [^{3}H]thymidine (2 μCi/well) during the final 18 h, after which the cells were harvested onto glass fiber filter mats using a Packard Micromate 196 cell harvester (Downers Grove, IL). Filter mats were sealed into plastic bags with 5 ml of betaplate scintillation fluid (Perkin-Elmer, Gaithersburg, MD) and counted using a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer). Samples were run in triplicate.

Stimulation Index (SI)

$$= \frac{(\text{cpm of transduced experimental tumor cells} + \text{allogeneic PBMC}) - (\text{cpm of transduced tumor cells alone})}{\text{cpm of allogeneic PBMCs alone}}$$

Responder PBMCs do not express DRB0101.

TT-boosted PBMCs. DRB0101 PBMCs (2×10^7 cells/4 ml/well) were cultured with 1 μg/ml of exogenous TT (Accurate, Westbury, NY) in 6-well tissue culture plates (Corning). After 5 days of culture, nonadherent cells were harvested, washed twice with Iscove's modified Dulbecco's medium, and replated in culture medium with 20 units/ml of recombinant human interleukin 2 (R&D Systems, Minneapolis, MN) at 1×10^6 cells/2 ml/well in 24-well plates (Corning). Remaining nonadherent cells were harvested 7 days later, and live cells were isolated using Histopaque-1077 separation medium. TT-activated, nonadherent cells were maintained in culture medium without exogenous interleukin 2 overnight and used the following day.

Antigen Presentation Assays for Endogenous TT and Exogenous TT Peptides. Irradiated (50 Gy) stimulator cells (1×10^4 or 2.5×10^4 cells/well) were cocultured in triplicate with adherent cell-depleted, TT-primed DRB0101 responder PBMCs (5×10^4 cells/well) in 200 μl/well in flat-bottomed 96-well microtiter plates (Corning). After 2 days of culture, supernatants were collected and assayed by ELISA for IFN-γ according to the manufacturer's instructions (Endogen, Woburn, MA). For the antibody blocking experiments, 1×10^4 stimulator cells were incubated with 10 μg/ml or 12.5 pg/ml of L243 (anti-HLA-DR) or 28.14.8 (isotype-matched irrelevant mAb) in 100 μl/well for 45 min before the addition of responder PBMCs. Values are the averages of triplicate points with their SDs.

For exogenous TT peptide p2 presentation, assays were as for endogenous antigen presentation, except soluble TT peptide p2 (22, 23) was added at the beginning of the 2-day culture period, and antigen-presenting cells not transduced with TT were used.

CD4, CD8, and CD19 Cell Depletions. Adherent cell-depleted, TT-primed PBMCs were depleted for $CD4^+$, $CD8^+$, or $CD19^+$ cells using magnetic beads, LD columns, and the QuadroMACS separation system according to the manufacturer's instructions (Miltenyi Biotech). Purity of depleted fractions was confirmed by flow cytometry.

HLA-DR Nomenclature. The PBMCs used in these studies were HLA typed by PCR; hence, they are known to be HLA-DRB0101. The HLA-DR gene used in these studies was sequenced and identified as HLA-DRB0101 and

[3] Internet address: www.cancer.med.umich.edu/breast_cell/umbnkdb.htm.

is abbreviated as "DR1" in the names of the transductants. The TT p2 peptide has been identified as a DR1-restricted epitope; however, its DR1 subtype is not known.

RESULTS

Construction of Retroviruses Encoding HLA-DRα Plus HLA-DRβ, CD80, and TT Fragment C. To generate human tumor cells expressing high levels of MHC class II molecules, retroviruses encoding HLA-DRα plus HLA-DRβ genes have been generated. The HLA-DRB0101 allele was selected because it is one of the more common alleles in the Caucasian population and is a frequently used restriction element (24, 25). A novel bicistronic retroviral vector that drives coordinate expression of approximately equimolar amounts of HLA-DRα and HLA-DRβ was developed using the pLNCX2(neo) retroviral backbone. DRα and DRβ0101 cDNAs (18) were cloned upstream and downstream, respectively, of the internal ribosomal entry site (IRES) of the vector pIRES. The DRA-pIRES-DRB segment was then excised from the pIRES vector and ligated into the pLNCX plasmid to yield the pLNCX/DRβ1 plasmid (Fig. 1*A*). This construct will produce a single-chain mRNA driven by the cytomegalovirus promoter in which DRα is translated by a CAP-dependent mechanism and DRβ is translated via the IRES in a CAP-independent manner.

Because of the critical role of costimulatory molecules in the activation of naive T cells (26), we have also generated retroviral plasmids encoding human CD80 (hCD80). The *hCD80* gene was excised from the pREP10/B7.1 plasmid and ligated into the retroviral vector pLHCX(HPH) or pLPCX(Puro) to form the pLHCX/CD80 (Fig. 1*B*) or pLPCX/CD80 (Fig. 1*C*) plasmids, respectively.

To monitor presentation of endogenously synthesized antigen, a retroviral plasmid encoding the TT fragment C was generated. The TT fragment C gene was excised from the pCR Blunt plasmid, an ATG start codon was inserted at its 5′ end, and the resulting construct was ligated into the pLPCX(Puro) vector to form the pLPCX/TT retroviral plasmid (Fig. 1*D*). All retroviral plasmids were packaged in 293T cells, and supernatants containing infectious retroviruses were harvested and used to transduce target tumor cells.

Transduced Human Tumor Cells Express Cell Surface HLA-DRB0101 and CD80 and Internal TT. The human ocular melanoma cell line Mel 202 and the mammary carcinoma cell line SUM159PT were transduced with different combinations of the pLNCX2/DR1, pLHCX/CD80, pLPCX/CD80, and pLPCX/TT retroviruses. The resulting transductants are shown in Table 1. SUM159PT and Mel 202 tumors were chosen because they do not constitutively express MHC class II molecules and hence should not express Ii, which we have shown previously inhibits presentation of MHC class II-restricted endogenous antigens (14, 17). To assess the magnitude and stability of transgene expression, transductants were tested by immunofluorescence and flow cytometry 1 week after being placed on drug selection (see Table 1 for drug selection conditions for each transductant line) and intermittently for 6 months thereafter. As shown in Fig. 2, Mel 202 and SUM159PT transductants express high levels of cell surface HLA-DR (L243 mAb), CD80 (CD80-PE mAb), and internal TT (polyclonal anti-TT ab), as measured at 6 months after transduction. HLA-DR-expressing Mel 202 and SUM159PT cells were also biotinylated, and the cell extracts were immunoprecipitated with anti-HLA-DR mAbs to assure proper structural conformation of cell surface-expressed, transduced class II molecules. Both lines displayed high levels of SDS-stable MHC class II $\alpha\beta$ dimers, indicating proper conformation and peptide binding.[4] The parental lines and transductants were also stained for MHC class I molecules (W6/32 mAb). All lines showed strong class I expression, with transductants displaying levels roughly comparable with their parental lines (data not shown).

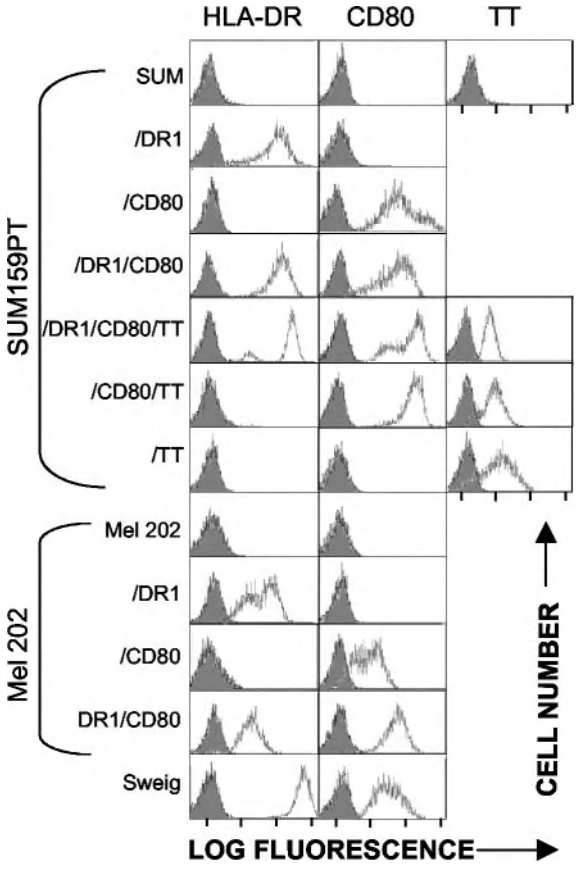

Fig. 2. SUM159PT and Mel 202 transductants express MHC class II HLA-DR and CD80 on the cell surface and TT internally. Live transductants were stained by direct immunofluorescence for plasma membrane HLA-DR (L243-FITC) or CD80 (CD80-PE). Fixed cells were stained by indirect immunofluorescence for internal TT (TT mAb plus fluorescent conjugate). *Gray peaks* denote staining with fluorescent conjugate alone or isotype control; *white peaks* represent staining with directly coupled primary antibody or primary antibody plus fluorescent conjugate. These data are from one of three to five independent experiments.

To ascertain that the MHC class II expression is allele specific, SUM/DR1 and SUM/DR1/CD80 cells were stained for cell surface expression of HLA-DR1 using the HLA-DR1-specific mAb. As shown in Fig. 3, pLNCX2/DR1-transduced SUM cells express high levels of DR1 and only stain at background levels with an irrelevant HLA-DR2-specific mAb. Therefore, SUM/DR1/CD80, SUM/DR1, SUM/CD80, SUM/DR1/CD80/TT, Mel 202/DR1, Mel 202/CD80, and Mel 202/DR1/CD80 transductants express high levels of the transduced *HLA-DR*, *CD80*, and/or *TT* genes as measured by antibody reactivity and immunofluorescence.

SUM159PT and Mel 202 Cells Do Not Express Invariant Chain. Because coexpression of Ii inhibits endogenous antigen presentation by MHC class II vaccine cells (14, 17), SUM159PT and Mel 202 cells were tested to ascertain that they do not express Ii. Cells were permeabilized, stained with the Ii-specific mAb PIN-1, and analyzed by flow cytometry. As shown in Fig. 4*A*, neither tumor line contains Ii, whereas the human B cell line, Sweig, which constitutively expresses Ii, is strongly positive. To further confirm the absence of Ii, detergent extracts of SUM159PT, Mel 202, Ii-positive Sweig, and Ii-negative Jurkat cells were electrophoresed by SDS-PAGE and analyzed by Western blotting for Ii expression. As shown in Fig. 4*B*, neither SUM159PT, Mel 202, Mel 202/DR1/CD80, nor SUM/DR1/

[4] V. Clements, unpublished results.

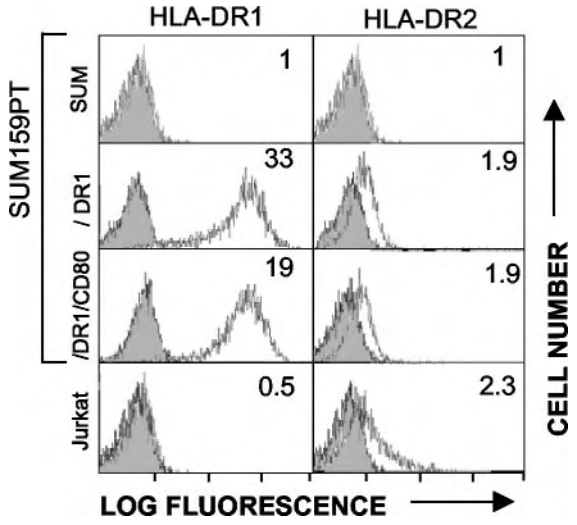

Fig. 3. SUM/DR1 and SUM/DR1/CD80 transductants express HLA-DR1 at the cell surface. Live cells were stained by indirect immunofluorescence for plasma membrane HLA-DR1 (mAb HLA-DR1 biotin) or with an irrelevant Ab (HLA-DR2-biotin) plus an avidin-PE conjugate. Jurkat is a DR1$^-$ cell line. *Gray peaks* denote staining with fluorescent conjugate without primary antibody; *white peaks* represent staining with primary antibody plus fluorescent conjugate. Numbers in the *upper right-hand corner* of each profile are the mean channel fluorescence for the antibody stained peak. These data are from one of two to five independent experiments.

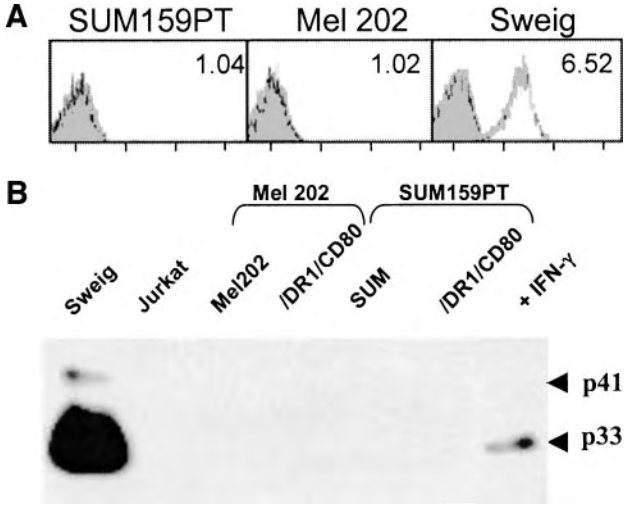

Fig. 4. Sum159PT and Mel 202 cells do not express Ii chain. *A*, fixed cells were stained by indirect immunofluorescence for Ii (mAb PIN1.1; *white peaks*) or with fluorescent conjugate alone (*gray peaks*). These data are representative of three independent experiments. *B*, uninduced or IFN-γ-treated (+ *IFN*γ) cells were detergent lysed, electrophoresed on 10% SDS-PAGE gels under reducing conditions, and transferred to nitrocellulose. Blots were stained for Ii with the mAb PIN1.1. Sweig and Jurkat cells are Ii$^+$ and Ii$^-$ cell lines, respectively. These data are from one of two to three independent experiments.

CD80 cells contain detectable Ii, although Ii expression is inducible in SUM159PT cells by a 48-h treatment with IFN-γ. Therefore, SUM159PT and Mel 202 tumor cells do not constitutively express Ii; therefore, Ii will not be present in the transduced vaccine cells to inhibit binding and presentation of endogenously synthesized peptides.

HLA-DRB0101 Transductants Stimulate HLA-DR Allogeneic PBMCs. Coculture of cells expressing functional HLA-DR molecules with allogeneic CD4$^+$ T lymphocytes results in T-cell proliferation (27). Therefore, to determine whether the HLA-DRB0101 molecules expressed by the transduced tumor cell vaccines are functional, we cocultured the various transductants with allogeneic PBMCs. Responder non-HLA-DRB0101 PBMCs were mixed with various numbers of irradiated transductants, and proliferation was assessed by measuring the SI at the end of 6 days of culture. Irradiated allogeneic PBMCs were used as a positive control. As shown in Fig. 5*A*, SUM/DR1/CD80 cells induce high SI, whereas SUM, SUM/CD80, or SUM/DR1 transductants produce only background levels. Therefore, the cell-based vaccines activate allogeneic PBMCs, provided they coexpress DRB0101 and CD80.

Transduced Tumor Cells Present an HLA-DR1-restricted TT Peptide. TT peptide p2 is an HLA-DR1-restricted epitope (22). If the HLA-DRB0101 molecules of the transductants are properly conformed and functional, when pulsed with the TT p2 peptide, the transductants should activate TT-specific HLA-DRB0101 lymphocytes. Because the TT-specific CD4$^+$ T-cell precursor frequency in peripheral blood of the DRB0101 donor was low (data not shown), the HLA-DRB0101 PBMCs were boosted *in vitro* with TT to expand the number of TT-reactive T cells. TT-boosted PBMCs were incubated at various ratios with tumor cell transductants pulsed with various quantities of TT p2 peptide to determine whether the transductants present this HLA-DR1-restricted epitope. T-cell activation was assessed by measuring IFN-γ release. As shown in Fig. 5*B*, SUM/DR1/CD80 tumor cells activate the TT-specific T cells as or more efficiently than EBV-transformed HLA-DR1 B cells (DR1-EBV B cells), whereas HLA-DR1-negative parental SUM cells do not activate. Therefore, SUM/DR1/CD80 tumor cells are effective APCs for an HLA-DR1-restricted epitope, further demonstrating that the transduced MHC class II molecules are functional.

HLA-DR1/CD80 Tumor Cell Transductants Present Endogenous TT and Activate TT-specific T Lymphocytes. We have generated the DR1/CD80 transductants to use as cancer vaccines to immunize patients and activate their T lymphocytes to tumor-encoded

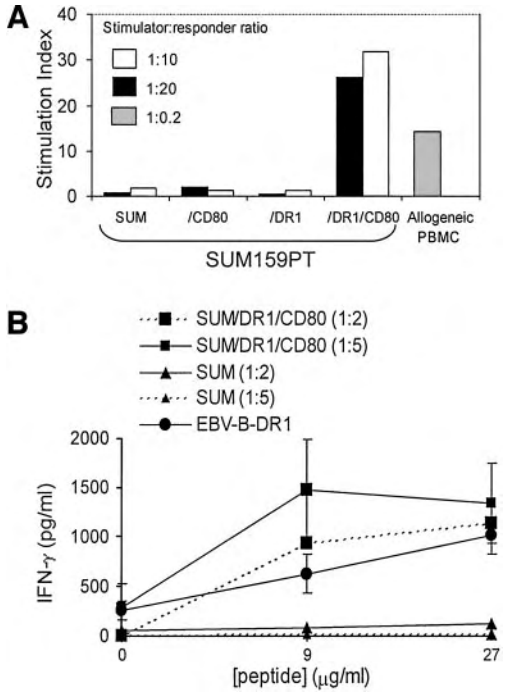

Fig. 5. SUM/DR1/CD80 cells induce proliferation of allogeneic T cells and present a DR1-restricted peptide to DRB0101 PBMC. *A*, irradiated SUM transductants expressing HLA-DRB0101 and/or CD80 or allogeneic PBMCs were cocultured with non-DRB0101 PBMCs at various ratios of APCs to responder lymphocytes. Proliferation was assessed by measuring the SI after 6 days of culture. These data are from one of three independent experiments. *B*, SUM, SUM/DR1/CD80, or DRB0101-expressing EBV B cells were pulsed with the DR1-restricted TT peptide, p2, and cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of three independent experiments. *Bars*, SD.

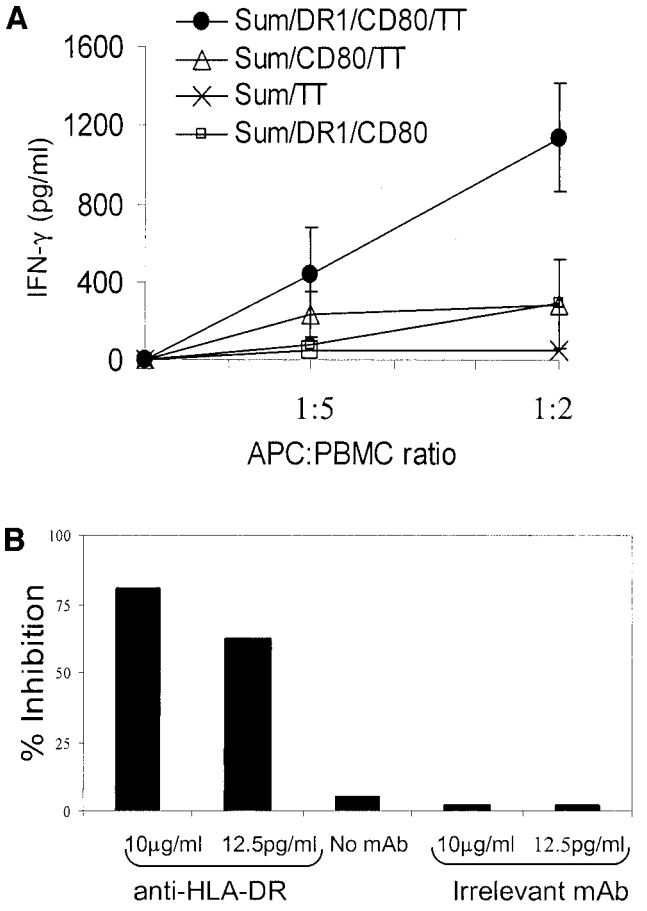

Fig. 6. SUM/DR1/CD80/TT cells activate HLA-DR-restricted DRB0101 PBMCs to tumor-encoded TT. *A,* irradiated SUM transductants were cocultured with TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of three independent experiments; *bars,* SD. *B,* HLA-DR-specific (L243) or irrelevant (28-14-8) mAb was added to culture wells containing irradiated SUM/DR1/CD80/TT cells before addition of TT-primed responder DRB0101 PBMCs at a ratio of 1:2 APCs to responder cells. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of two independent experiments.

tumor peptides. To achieve this goal, the transductants must not only express functional HLA-DR molecules, but the DR molecules must also bind and present endogenously synthesized tumor peptides. To determine whether the transductants have this capability, we tested SUM/DR1/CD80/TT cells as APCs for endogenously encoded TT. Because the TT construct does not contain a signal sequence, TT protein will reside in the cytoplasm and serve as a "model" tumor antigen for a cytoplasmically localized tumor antigen.

Adherent cell-depleted HLA-DRB0101 PBMCs were boosted *in vitro* with TT as per the experiment of Fig. 5*B* and cocultured at various ratios with irradiated transduced SUM cells. Activation was assessed by measuring IFN-γ release. As shown in Fig. 6*A,* SUM/DR1/CD80/TT tumor cells activate a potent T-cell response, whereas SUM transductants without DRB0101 (SUM/CD80/TT), without TT (SUM/DR1/CD80), or without DRB0101 and CD80 (SUM/TT) do not activate. Because SUM/TT and SUM/CD80/TT cells do not activate, TT is not being released into the culture medium and being presented by other APCs in the PBMC population. Therefore, tumor cells transduced with *HLA-DRB0101, CD80,* and *TT* genes are effective APCs for endogenously encoded molecules.

To further analyze whether the presentation of endogenous TT is DR1 restricted, anti-HLA-DR mAb (L243) was added at various concentrations at the beginning of the assay. As shown in Fig. 6*B,* in the presence of the highest dose of antibody, T-cell activation is inhibited >80%, whereas an irrelevant isotype-matched mouse H-2L^d-specific mAb does not inhibit.

DR1/CD80/TT Tumor Cells Activate CD4+ T Lymphocytes. To identify the PBMCs that are specifically activated by the vaccine cells, adherent cell-depleted, TT-primed DRB0101 PBMCs were depleted for CD4+ or CD8+ T cells or for B cells and then used as responding cells in antigen presentation assays with SUM/DR1/CD80/TT transductants. T and B cells were depleted by magnetic bead separation. To ascertain the efficiency of the depletions, PBMCs before and after depletion were tested by flow cytometry for the percentage of CD4+, CD8+, and immunoglobulin+ (B) cells. As shown in Fig. 7*A,* antibody depletion eliminated 98–99% of the target lymphocytes. The relatively high percentage of CD4+ T cells and low percentage of CD8+ T cells in the undepleted, TT-boosted population probably reflects the preferential activation of CD4+ T cells during the *in vitro* boosting process.

After T- and B-cell depletion, the resulting PBMCs were cocultured with irradiated vaccine cells and endogenous TT presentation assessed by ELISA. As shown in Fig. 7*B,* CD4-depleted PBMCs stimulated with SUM159/DR1/CD80/TT vaccine cells are not activated, as measured by IFN-γ release. In contrast, CD8-depletion did not affect IFN-γ release. Likewise, depletion of CD19+ cells did not affect IFN-γ release, demonstrating that cross-priming by B cells is not occurring. Stimulation of undepleted PBMCs with SUM159/DR1/CD80 APCs also did not cause IFN-γ release, demonstrating that

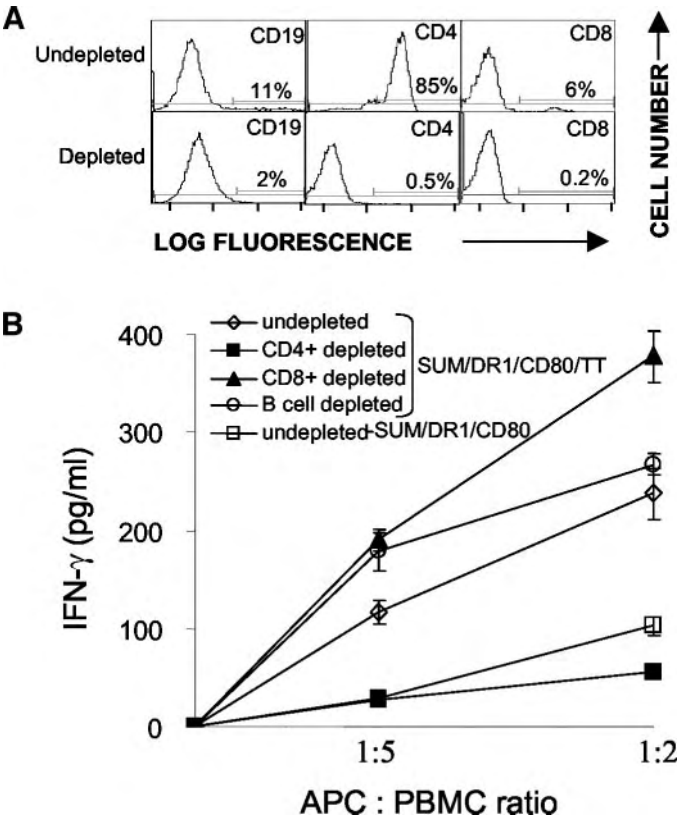

Fig. 7. SUM/DR1/CD80/TT cells activate CD4+ T lymphocytes to tumor-encoded antigen. *A,* DRB0101 PBMCs were primed *in vitro* with TT, and separate aliquots were depleted for CD4+, CD8+, or CD19+ cells. The resulting cells were stained by direct immunofluorescence for these populations. Values in the *lower right-hand corners* of each profile represent the percentage of the indicated cells. *B,* irradiated SUM/DR1/CD80/TT or SUM/DR1/CD80/transductants were cocultured with CD4, CD8, or CD19-depleted, or not depleted, TT-primed DRB0101 PBMCs at various ratios of APCs to responder PBMCs. Supernatants were harvested after 2 days of culture and tested by ELISA for IFN-γ. These data are from one of two to four independent experiments; *bars,* SD.

PBMC activation is TT specific. Therefore, $CD4^+$ PBMCs are activated by the vaccine cells, and the activation is mediated by direct presentation of endogenously synthesized TT by the genetically modified tumor cell transductants.

DISCUSSION

Recent animal studies and some clinical trials have indicated that the use of genetically engineered tumor cells as vaccines may have therapeutic efficacy for the treatment of cancer (28–30). Parallel studies have recognized the critical role played by $CD4^+$ T cells in orchestrating the host immune response against cancer and have developed methods to activate $CD4^+$ T cells (2–5, 31–33). Because $CD4^+$ T cells play a central role in enhancing antitumor immunity, our laboratory has focused on facilitating the activation of these cells. We have hypothesized that tumor cells that constitutively express MHC class I molecules do not contain Ii and are genetically modified to express syngeneic MHC class II molecules, and costimulatory molecules will function as APCs for endogenously synthesized MHC class I- and class II-restricted tumor antigen epitopes. If used as immunogens in tumor-bearing individuals, such cells will serve as "vaccines" to activate tumor-specific $CD4^+$ and $CD8^+$ T lymphocytes that will facilitate regression of wild-type tumor (2, 6). Because the efficacy of these vaccines against wild-type primary tumors and experimental and spontaneous metastatic disease has been demonstrated in multiple mouse models (8, 10, 11, 34, 35), the goal of this study was to translate this strategy for clinical use.

Activation of tumor-specific $CD4^+$ T cells by the cell-based vaccines is based on the supposition that the MHC class II molecules of the vaccine cells bind peptides synthesized within the tumor cells and directly present these peptides to $CD4^+$ T lymphocytes. This mode of presentation is different from that of professional APCs that typically bind peptides derived from endocytosed, exogenously synthesized antigens (36). This fundamental difference is attributable to the absence of the MHC class II-associated accessory molecule, Ii, in the vaccine cells. If APCs express Ii, Ii binds to newly synthesized MHC class II molecules, thereby preventing the binding of endogenously derived peptides and favoring the binding of exogenously synthesized peptides (37). However, in the absence of Ii, MHC class II molecules bind peptides derived from endogenously synthesized antigens (14). Because the MHC class II and Ii genes are coordinately regulated and coordinately induced by IFN-γ (38), professional APCs and tumor cells that constitutively express MHC class II genes and/or are induced by IFN-γ are unlikely to be APCs for endogenously synthesized tumor antigens. Studies with Ii^+ and Ii^- MHC class II^+ tumor cells support this concept and demonstrate that the most efficacious vaccines are MHC class II^+Ii^- (14, 16, 17).

Early studies suggested that expression of MHC class II molecules without coexpression of Ii produces reduced levels of class II molecules that are improperly conformed and unable to function as antigen presentation elements (39–41). More recent studies have demonstrated that the Ii dependency of MHC class II molecules is allele specific (42, 43), and that many MHC class II alleles do not require Ii expression for stability or antigen presentation function (44). The studies reported here demonstrating efficient antigen presentation by MHC class II^+Ii^- tumor cell vaccines add HLA-DR0101 to the list of MHC class II alleles whose expression and function are independent of Ii coexpression.

In addition to the absence of Ii for maximal vaccine efficacy, the studies reported here demonstrate that optimal vaccine activity requires coexpression of CD80 for delivery of a costimulatory signal. This observation agrees with extensive mouse and human studies showing the requirement for costimulation for optimal T-cell activation (reviewed in Ref. 26), as well as many studies that showed that CD80 expression facilitates tumor rejection (45–47).

Several lines of evidence support the hypothesis that the MHC class II tumor cell-based vaccines activate $CD4^+$ T cells by direct antigen presentation of endogenously encoded tumor antigens, rather than by cross-priming or indirect presentation via host-derived APCs, as suggested by other investigators for other cell-based vaccines and/or tumor cells (48–50):

(*a*) If tumor-encoded antigens were presented by host-derived APCs such as B cells or other APCs in the PBMCs, then SUM/DR1/TT, SUM/TT, and SUM/CD80/TT cell lines should be just as effective APCs as are SUM/DR1/CD80/TT. However, only SUM/DR1/CD80/TT vaccine cells activate PBMCs.

(*b*) If professional APCs, rather than the tumor cell vaccines, are the relevant APCs, then removal of these professional APCs should eliminate T-cell activation. However, adherent cells (including dendritic cells and macrophages) are routinely removed from the PBMCs before their coculture with vaccine cells, and in some experiments, $CD19^+$ B cells were also removed without affecting T-cell activation.

(*c*) Extensive *in vivo* studies using genetically marked vaccine cells conclusively demonstrated that the vaccine cells directly activate T lymphocytes (12–14). Therefore, it is unlikely that vaccine efficacy is attributable to leakage of tumor antigen, resulting in endocytosis by professional APCs for presentation by cross-priming.

The vaccines described here are based on the premise that tumor cells will be destroyed by $CD8^+$ T cells with help from $CD4^+$ T cells. Tumor-specific $CD8^+$ T cells could be activated either by interacting with MHC class I/peptide complexes of the genetically modified vaccine cells or by cross-presentation of class I-restricted epitopes by professional APCs. In either case, the activated $CD8^+$ T cells would be specific for MHC class I-restricted tumor peptides and for wild-type tumor cells. Although the vaccines described here are potent activators of $CD4^+$ T cells, vaccine cell expression of a MHC class I allele shared with the patient's lymphocytes may facilitate an even stronger immune response by capitalizing on the close proximity of $CD4^+$ and $CD8^+$ T cells during their activation. A MHC class I allele could be expressed in the vaccines by retroviral transduction. Alternatively, for an allele such as HLA-A2, which is expressed by approximately 50% of the Caucasian population, an HLA-$A2^+$ tumor cell line could be used as the "base" vaccine. Additional experiments assessing activation of $CD8^+$ T cells by the vaccines generated in this study *versus* MHC class I-matched or -engineered vaccines will be necessary to address this issue.

A significant technical obstacle in generating the MHC class II cell-based vaccines has been to routinely achieve high level expression of the desired MHC class II alleles in human tumor cells. Because many human tumor cells and cell lines can be problematic to maintain in culture, standard transfection and electroporation techniques did not result in reproducible class II expression.[5] In contrast, transduction using a bicistronic retrovirus encoding the DRα and DRβ chain genes separated by an IRES routinely yielded high-level HLA-DR expression in a high proportion of transductants. The efficiency of the current retroviruses appears to be attributable to the placement of the DRα and DRβ genes flanking the IRES, because a previous study using a retroviral construct encoding pig DQα and DQβ genes run off of separate promoters and without an IRES produced only low-level, DQ-expressing cells (51). It is likely the IRES construct will be universally useful, because similar retroviruses encoding other HLA-DR alleles also reproducibly yield high-level MHC class II expression in additional human tumor lines.[6]

The potency of the MHC class II vaccines for activating $CD4^+$ T cells

[5] S. Dissanayake and J. Bosch, unpublished results.
[6] J. Thompson and M. Pohl, unpublished results.

to tumor-encoded antigens suggests that these vaccines may have therapeutic efficacy for cancer patients. For example, the cell-based vaccines could be administered *in vivo* to patients with disseminated metastatic disease. Alternatively, they could be used *ex vivo* to activate patients' T cells for subsequent adoptive transfer. In either case, these vaccines provide a novel and potent approach for activating tumor-specific $CD4^+$ T cells and merit further clinical development and testing.

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Approval Sheet

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The MHC class II associated invariant chain limits the immunogenicity of genetically modified human tumor cell vaccines.

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Peer-reviwed Publications:

- James A. Thompson, Samudra K. Dissanayake, Keith L. Knutson, Mary N. Disis, and Suzanne Ostrand-Rosenberg. *Tumor Cells Transduced With the MHC Class II Transactivator Activate Tumor-Specific CD4$^+$ T cells Whether or Not They are Silenced for Invariant Chain*. Cancer Res. 2006 66: 1147-1154.
- Jacobus J. Bosch, James A. Thompson, Minu K. Srivastava, Uzoma K. Iheagwara, Timothy G. Murray, Michal Lotem, Bruce R. Ksander, and Suzanne Ostrand-Rosenberg. *MHC II Transduced Tumor Cells Originating in the Immune Privileged Eye Prime and Boost CD4$^+$ T Lymphocytes That Cross-react with Primary and Metastatic Uveal Melanoma Cells.* Cancer Res. 2007 In Print
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- Thompson, J. A., Dissanayake S. K., Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA interference (RNAi) to down regulate invariant chain. Department of Defense Breast Cancer Research Program Meeting: Era of Hope, Philadelphia, PA, USA; 2005
- Thompson, J. A. & Ostrand-Rosenberg S., Novel MHC class II breast cancer vaccine using RNA Interference (RNAi) to down regulate invariant chain: An MHC II+ Ii- tumor vaccine approach. University of Maryland Graduate Student Association of Biological Sciences (GABS) Symposium, Baltimore, MD, USA; 2004

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Abstract

The MHC class II associated invariant chain limits the immunogenicity of genetically modified human tumor cell vaccines.

James Thompson, Doctor of Philosophy, 2007

Thesis Directed by: Suzanne Ostrand-Rosenberg, Professor, Biological Sciences

Activation of tumor-reactive T lymphocytes is a promising approach for the treatment of patients with metastatic cancers. $CD8^+$ T cells can have cytotoxicity and specificity for tumor cells and optimal $CD8^+$ T cell activity and memory requires $CD4^+$ T cell co-activation. Therefore, we are developing "MHC-II" vaccines that activate tumor-reactive $CD4^+$ T cells. MHC-II vaccines are MHC class I^+ tumor cells transduced with costimulatory molecules and MHC-II alleles syngeneic to the prospective recipient. In previous studies mouse MHC-II vaccines were therapeutic for established mouse tumors, provided they did not co-express the class II-associated Invariant chain (Ii), an accessory molecule coordinately regulated with MHC II. A major goal of my thesis has been to develop human MHC-II vaccines. I hypothesized that in the absence of Ii, MHC-II presents novel *endogenously* synthesized tumor peptides not presented by professional Ii^+ antigen presenting cells (APC), thereby circumventing existing antigen-specific tumor-induced tolerance. To express a single MHC-II allele in the absence of Ii, MHC-II^-Ii^- cells were transduced with a bicistronic retroviral vector which expresses the MHC-II α and β genes. To express multiple MHC-II alleles and to facilitate presentation of a broader repertoire of tumor antigens, tumor cells were transduced with the MHC-II transactivator (CIITA), a regulatory gene that coordinately increases expression of all MHC-II alleles.

RNAi expression vectors silenced Ii >95% in CIITA/CD80/siRNA transductants. Down-regulation of Ii did not affect MHC-II expression or stability, and Ii^+ and Ii^- transductants activate human $CD4^+$ T cells to DRB1*0701-restricted HER2/neu epitopes. HER2/neu^+ MHC-II^+ non-malignant cells (MCF10A) did not activate HER2/neu specific $CD4^+$ T cells, suggesting tumor vaccines may not affect non-malignant tissues. MHC-II vaccines are more efficient than Ii^+ APC for priming and boosting tumor-specific Type 1 $CD4^+$ T cells, and they induce greater expansion of $CD4^+$ T cells which secrete more IFNγ and activate an overlapping, but distinct, repertoire of $CD4^+$ T cells. Therefore, MHC-II vaccines facilitate a robust $CD4^+$ T cell response that includes the presentation of peptides that are uniquely presented by Ii^- cells. These vaccines may be unique agents which induce a strong tumor-specific immune response in patients.

The MHC class II associated invariant chain limits the immunogenicity of genetically modified human tumor cell vaccines

by
James Andrew Thompson

Thesis submitted to the Faculty of the Graduate School
of the University of Maryland in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2007

Dedication

I dedicate this dissertation to my wife Gwen who stood by me and deserves a doctorate for her efforts in helping me to achieve my goals; my three boys, Spencer, Benjamin and Jameson for being without a father for many months and still loving me when I came home; my mother for always believing I would do great things; and to my father who did not live to see this day but continues to be a part of my life and a part of why I have finished my doctorate.

Table of Contents

Chapter 1

Introduction

Chapter1: Introduction

This thesis project focused on studying what affect the class II MHC associated invariant chain (Ii) has on genetically modified tumor vaccines. Work done prior to this thesis has shown that mouse tumor cells genetically modified to express an immune modulator molecule, class II major histocompatibility molecule (MHC II), can be used as therapeutic agents for the treatment of cancer (3-5). Immunotherapy studies with mammary carcinoma and sarcoma in mice have shown that MHC II^+ cell-based tumor vaccines significantly reduce primary tumor and/or metastatic disease and can extend survival time (3, 5-10). Tumor vaccines using MHC II^+ genetically modified tumor cells as therapeutic agents are only effective in the absence of Ii. I hypothesize that (1) Ii limits the immunogenic factors (antigens) presented by tumors to only those which activate T cells with low affinity and that (2) in the absence of Ii tumor cells present different antigens which may activate T cells of higher affinity. Clinical findings showing that high levels of cell surface Ii in MHC II^+ tumors is a predictor of poor prognosis, supports our hypothesis that Ii inhibits the ability of MHC II^+ tumors to induce a strong immune response (11, 12). I have genetically modified tumor cells to express MHC II in the presence and absence of Ii in order to study the effect of Ii on tumor immunogenicity. I provide evidence in the body of this thesis to support my hypothesis that MHC II^+ Ii^- tumor cells activate a separate set of T cells with higher affinity, than those T cells activated by MHC II^+ Ii^+ tumor cells. This introductory chapter will provide the background information for this project.

A. Primary tumors are often treatable by resection but most patients die from metastatic disease.

If found early, while only localized primary tumors exist, some cancers, such as breast cancer, are treatable with existing therapies, but for patients who develop metastasis there are currently few if any cures. Cancer is the second leading cause of death in the United States. In 2003, 22.7% of mortalities in America were caused by cancer, killing 556,902 Americans (13). Breast cancer is the leading cause of cancer in women in the US, with 212,920 estimated new cases being diagnosed in 2006 (14). Breast cancer is the second leading cause of cancer death in women in the US, killing 40,970 women in 2006 (14). One in eight women in the world will acquire breast cancer in their lifetime (15). Primary breast cancer is treatable by surgical resection and itself is not life threatening. Deaths from breast cancer are due to cancer metastasis to distant sites such as lung, bone, liver, and brain. Unfortunately in cases where breast cancer has metastasized to distant organs, other than the lymph nodes under the arms (Stage IV), only 25% of patients will survive past 5 years (16) and only 10% of patients will survive past 10 years with current treatments (17). There are no current cures for stage IV metastatic breast cancer (18). The immune system provides a promising tool for treating metastasis. Tumor vaccines which induce an immune response against tumor have been shown to reduce metastasis and increase survival in mice with cancer (7).

B. Tumors are immunogenic and targeted therapies which enhance immune responses against tumors may eliminate primary tumor and metastasis.

The immune system is capable of recognizing and eliminating tumor cells, which are believed to arise continually. In 1909 Paul Ehrlich proposed that the body's own immune system might be able to eliminate cancer cells (19). This idea went untested until the 1950s when studies in inbred strains of mice demonstrated that mice could be immunized against syngeneic

transplants of tumors led both Sir Macfarlane Burnet and Lewis Thomas to propose the "cancer immunosurveillance" hypothesis. Both hypothesized that lymphocytes played a role in continually recognizing and eliminating transformed cells that would otherwise become malignant, and only when they failed would cancer arise (20, 21).

Studies where elements of the immune system are impaired or knocked out support the immunosurveillance theory. Blocking or knocking out IFNγ, a key cytokine for activation of immune cells such as T cells, contributed to an increase in chemically induced tumors (22-26). Knocking out the gene for perforin, needed by T cells and NK cells to kill targets, led to increased tumorigenicity (22, 25, 27-30). Definitive proof that lymphocytes are needed for tumor immunosurveillance came when comparing immune competent mice to mice knocked out for a gene (RAG-1 or -2) which is essential for recombination/ rearrangement of lymphocyte antigen receptors. Knocking out RAG genes led to complete loss of natural killer T (NKT), T and B cells. The RAG knock out mice were more susceptible to chemical carcinogen induced tumors and had increases in spontaneous neoplasia (31, 32). Looking at the contribution of different immune cell subsets, Girardi *et al.* studied mice deficient for T cells alone and found that T cells were important for protecting against cancer (33). Table I summarizes data that is consistent with immunosurveillance in mice deficient for specific components of the immune system, strengthening the theory that the immune system can play a role in cancer prevention and possibly cancer elimination.

If the immune system helps prevent cancer, can the immune system be harnessed to remove existing malignancies? Studies by Klein et al showed that tumors contained tumor infiltrating lymphocytes (TIL) made up largely of T cells (34). In most cases, increased TILs correlated with better prognosis (35). Use of tumor specific T cells as a therapeutic agent for treating tumor metastasis has great promise since T lymphocytes can have long-term memory and can recognize and destroy tumor cells without harming non-malignant, normal tissue. Boon and his colleagues gave early evidence of this, but also described that tumor was able to block T cell immunosurveillance (36). If this blocking of immunosurveillance or 'tumor induced tolerance' can be overcome and tumor specific T cells could be activated, it is possible that the activated T cells would remove existing tumor and protect against further metastasis.

Table I Enhanced susceptibility of immuno-deficient mice to formation of chemically induced and spontaneous tumors. Dunn et al 2002 (2)

Phenotype or depletion	Immunodeficiency	Tumor susceptibility
RAG-2$^{-/-}$	T, B and NKT cells	MCA-induced sarcmas. Spontaneous intestinal neoplasia.
RAG-2$^{-/-}$ x STAT I$^{-/-}$ (RkSk)	T, B and NKT cells Insensitive to IFN-γ and IFN-α/β	MCA-induced sarcomas. Spontaneous intestinal and mammary neoplasia.
BALB/c SCID	T, B and NKT cells	MCA-induced sarcomas.
Perforin$^{-/-}$	Lack of perforin	MCA-induced sarcomas. Spontaneous disseminated lymphomas.
TCR Jα281$^{-/-}$	Subset of NKT cells	MCA-induced sarcomas.
Anti-asialo-GMI antibody	NK cells and activated macrophages	MCA-induced sarcomas.
Anti-NK I.I antibody	NK and NKT cells	MCA-induced sarcomas.
Anti-Thyl antibody	T cells	MCA-induced sarcomas.
$\alpha\beta$ T cell$^{-/-}$	$\alpha\beta$ T cells	MCA-induced sarcomas.
$\gamma\delta$ T cell$^{-/-}$	$\gamma\delta$ T cells	MCA-induced sarcomas. DMBA/TPA-induced skin tumors.
STAT I$^{-/-}$	Insensitive to IFN-γ and IFN-α/β	MCA-induced sarcomas. Wider tumor spectrum in STAT I$^{-/-}$ STAT I$^{-/-}$ x p53$^{-/-}$.
IFNGRI receptor$^{-/-}$	Insensitive to IFN-γ	MCA-induced sarcomas. Wider tumor spectrum in IFN-γ receptor$^{-/-}$ x p53$^{-/-}$
IFN-$\gamma^{-/-}$	Lack of IFN-γ	MCA-induced sarcomas. C57BL/6: Spontaneous disseminated lymphomas. BALB/c: Spontaneous lung adenocarcinoma.
Perforin$^{-/-}$ + IFN-$\gamma^{-/-}$	Lack of performing and IFN-γ	MCA-induced sarcomas. Spontaneous disseminated lymphomas.
IL-12$^{-/-}$	Lack of IL-12	MCA-induced sarcomas.
WT + IL-12	Exogenous IL-12	Lower incidence of MCA-induced sarcomas.

Methylcholanthrene-treated wild-type (WT) mice were treated with IL-12 during tumor formation.

Many of the immune cells involved in tumor immunosurveillance (T cells) recognize and target cells which express immunogenic peptides (antigens). The next section describes how immune cells differentiate between normal tissue and tumor cells.

C. The immune system can target tumor cells because tumors express immunogenic peptides.

Tumors express many types of immunogenic peptides called “tumor antigens”, which activate T cells. (37, 38). Tumor antigens are any gene product expressed by tumors which can induce an immune response. Immunogenic tumor antigens may be unique antigens only expressed in tumor cells or antigens also present in other tissues but expressed at higher levels in tumor cells than normal cells. The following categories describe types of peptides expressed by tumors which have been found to induce an immune response:

Differentiation antigens are antigens which are shared between tumors and normal tissues from which the tumors arose. These antigens are derived from tissue specific genes such as genes involved in melanin biosynthesis shared by skin cells and melanoma. Therapies which activate T cells specific for differentiation antigens, even though they may induce T cell responses to healthy tissue as well as tumor, may still be more specific and less invasive than many existing chemotherapies used.

Over-expressed tumor antigens are those which are expressed in normal tissues, but at lower levels than in tumors. Because these antigens are expressed by normal tissue T cells which respond to these antigens usually have low avidity and may be tolerant to those antigens (39, 40). Due to high expression, antigens from alternatively spliced genes may become more prevalent leading to ‘cryptic epitopes’. Cryptic epitopes are novel peptides not normally

expressed which form from non-spliced introns, alternative open reading frames (ORF) or alternative post-translational splicing (41). Cryptic epitopes may become more prevalent as a protein is expressed at higher levels, which commonly occurs in tumors. Therefore, tumors which over express self proteins, may induce an immune response against those proteins.

Tumor-specific antigens are antigens that arise in tumors due to point mutations or splicing aberrations in genes caused by tumors' mutable nature (42). Tumor-specific antigens are more immunogenic than self antigens (43). The presence of known tumor specific antigens is a strong predictor of positive clinical outcome in cancer patients (44-46). These antigens seem to be promising targets for activating a strong tumor specific immune response, but unfortunately few are shared between tumors. Only those point mutations which occur in tumor suppressor genes and contribute to cancer are shared. Because tumor specific antigens may be unique to a specific patient, vaccines targeting such antigens would have to be prepared for individual patients.

Viral antigens are derived from viral gene products. Viruses are known to be associated with various cancers including: Burkitts lymphoma associated with Epstein-Barr virus (47); hepatocellular carcinoma associated with hepatitis B and C viruses (48, 49) and cervical carcinoma associated with human papilloma virus (50). Because viral antigens are non self antigens, therapies which activate an immune response against cells expressing viral antigens associated with cancer would only affect tumor cells and not normal tissue.

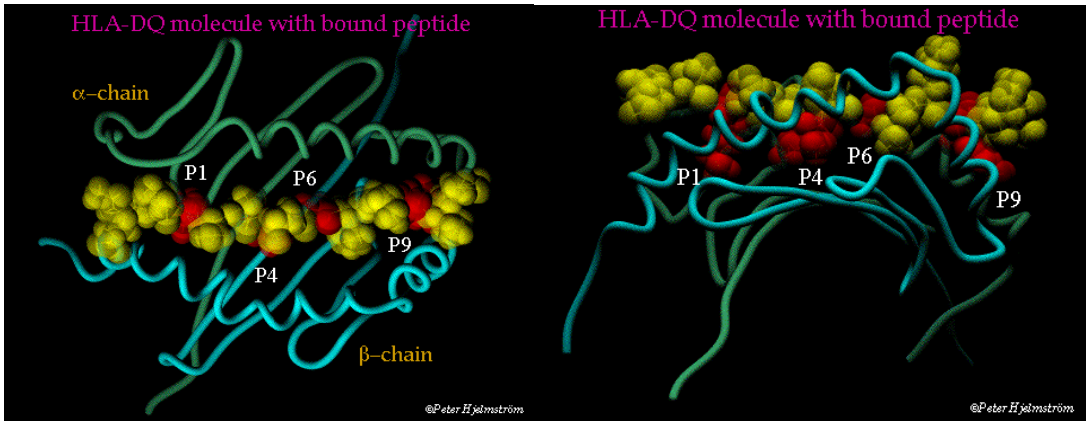
Tumor cells express a wide array of possible immunogenic peptides. Immune cells (T cells) differentiate between normal 'healthy tissue' and tumor cells based on what peptides are presented and it is the 'job' of MHC molecules to present potentially immunogenic peptides to T

cells. The next section describes MHC molecules and how they present such a wide array of peptides to T cells.

D. MHC molecules present immunogenic peptides to T cells.

T cells are activated when their dimeric T cell receptor recognizes and binds to peptides bound to class I or II MHC molecules also known as the Human Leukocyte Antigens (HLA). MHC molecules are heterodimeric molecules which form 'antigen binding grooves' that bind to a broad spectrum of peptides. MHC I has a closed ended peptide binding groove which binds 8-9 amino acid long peptides. MHC II has an open ended binding groove which can accommodate longer 13-18 amino acid long peptides which may extend out past the groove at either end **(figure 1)**. Each MHC allele binds peptides, which contain two to three particular amino acid 'anchor residues' at specific sites within the peptide. The peptides amino acid side chains interact with complementary 'pockets' in the MHC binding groove. Additional binding energy is provided by hydrophobic interactions and hydrogen bonding between the amino acids of the MHC I binding groove and the peptide. Even with these few restrictions to binding peptide, a wide array of possible peptides can bind to one MHC allele. Because each individual has multiple alleles of each MHC I and II gene and humans have 3 major MHC I genes and 3 major MHC II genes, the diversity of antigens that can be presented is huge. It is this ability of the MHC to present such a wide array of random peptides which allows the immune system to recognize the ever changing array of pathogens it encounters.

Figure 1. MHC II structure.
http://depts.washington.edu/rhwlab/resMat/dq/3structure.html

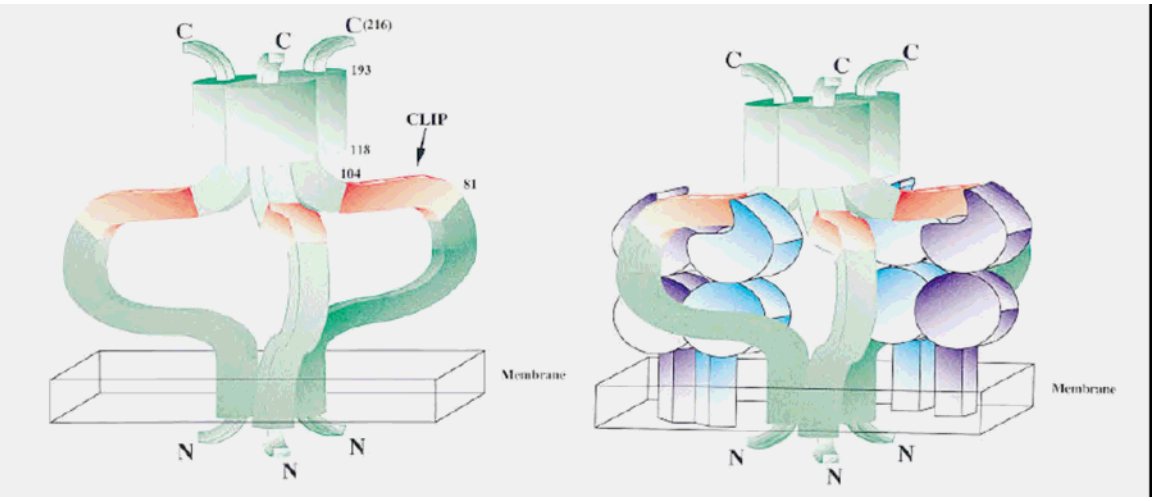


Class I MHC molecules are expressed on all nucleated cells in the body and bind to peptides processed from intracellular "endogenous" proteins. In class I MHC presentation of antigen, proteins that are recycled through the ubiquitin pathway are targeted to the proteosome and broken down into small 9-15 amino acid peptides. These peptides are brought into the ER through an ATP dependent mechanism, called transporter associated with antigen processing (TAP). Peptides are further cut into 8-9 amino acid lengths by the ER aminopeptidase ERAP1. These 8-9 amino acid long peptides bind to MHC I, which then traffics to the cell surface where it presents peptides to T cells.

In order to protect against activation of T cells to self antigens MHC II is only constitutively expressed on specialized professional antigen presenting cells (APC), such as dendritic cells (DC), B cells and macrophages. MHC II expression is regulated by a transcription factor called the class II trans-activator (CIITA). CIITA is constitutively expressed in professional APC and is also inducible in many other cell types through the IFN-γ pathway. IFN-γ is a cytokine secreted by immune cells in response to cell damage or infection. The α and β subunits of MHC II are synthesized in the ER where they normally associate with a type II (N-

terminus in cytosol) transmembrane glycoprotein chaperone molecule, MHC II associated invariant chain (Ii) (1, 51). MHC II presents mainly endocytosed, exogenous antigens because MHC II binds with high affinity to Ii and therefore Ii blocks loading of other endogenous peptides until Ii is degraded. Ii expression is also under the control of CIITA and is therefore almost always co-expressed with MHC II. Ii stabilizes the MHC II heterodimer; blocks the antigen binding groove, preventing the binding of ER resident proteins; and aids in trafficking MHC II to the endocytic pathway (52). MHC II forms nonamers with Ii where three Ii molecules bind to three MHC II αβ dimers and traffic together. This nonamer is depicted in figure 2. The MHC II-Ii complex traffics from the ER, through the trans golgi network (TGN), to endosomes, then to antigen-processing compartments also known as MHC II compartments (MIIC) (53).

Figure 2. Invariant chain and MHC II interactions
Figure from Cresswell et al (1)
Ii forms trimers which bind to three sets of MHC II dimers forming a nonameric complex. Only two MHC II dimers are shown in the figure in order to better see the binding of the other two MHC II dimers to the CLIP portion of each Ii molecule.



In the acidic MIICs, Ii is degraded by cathepsins (group of low pH functioning Proteases) leaving a short peptide fragment, the class II associated invariant peptide (CLIP) taking up the antigen binding groove (54). Acidic conditions lower the affinity for peptide to the MHC II antigen binding groove allowing for easier replacement of CLIP or other low affinity proteins for higher affinity antigenic peptides (1). Within these late endosomes another MHC accessory molecule HLA-DM, also up regulated by CIITA, facilitates the removal of CLIP and 'edits' the peptides that bind to MHC II (55, 56). Concurrently, exogenous antigens are endocytosed into the cell and trafficked into MIICs where they are degraded by acidic proteases and bind to the MHC II binding groove. In the presence of Ii mostly extracellular peptides are presented because the MHC II antigen binding groove is blocked by Ii until reaching the MIIC where exogenous peptides are at high concentrations. MHC II then traffics to the cell surface where it can 'present' the MHC II-antigen complex to $CD4^+$ T cells. A second pathway for MHC II is one where MHC II-Ii traffics directly to the cell surface from the TGN. These MHC II molecules are still bound to Ii, are endocytosed, then enter into the MIIC where Ii can be degraded and MHC II can bind to peptide and can again traffic to the cell surface (57). Likewise, mature MHCII-peptide complexes at the cell surface are recycled back into the MIIC where they may exchange peptides and return to the cell surface (58-60). This process is summarized in figure 3. Even though MHC II predominately presents peptides derived from exogenous proteins, MHC II has also been shown to present peptides from endogenously synthesized proteins (61). Nimmerjahn et al give evidence that autophagy may be involved in the presentation of peptides derived from some cytosolic proteins via MHC II (62). They proposed that endogenous proteins may enter endocytic compartments via autophagy where they are degraded and loaded onto MHC II.

The ability of cells to present a large array of peptides on their cell surface provides a platform by which immune cells can monitor the body for non self and or out of place protein expression. In general MHC I presents mainly endogenous peptides and MHC II on professional APC presents peptides phagocytosed or endocytosed from their surroundings. Although MHC I has been shown to present some exogenous peptides in DC during cross priming and MHC II has been shown to present endogenous peptides as well. T cells have receptors which bind to these MHC-peptide complexes and are activated when they bind with high affinity. The activation of T cells is described in the next section.

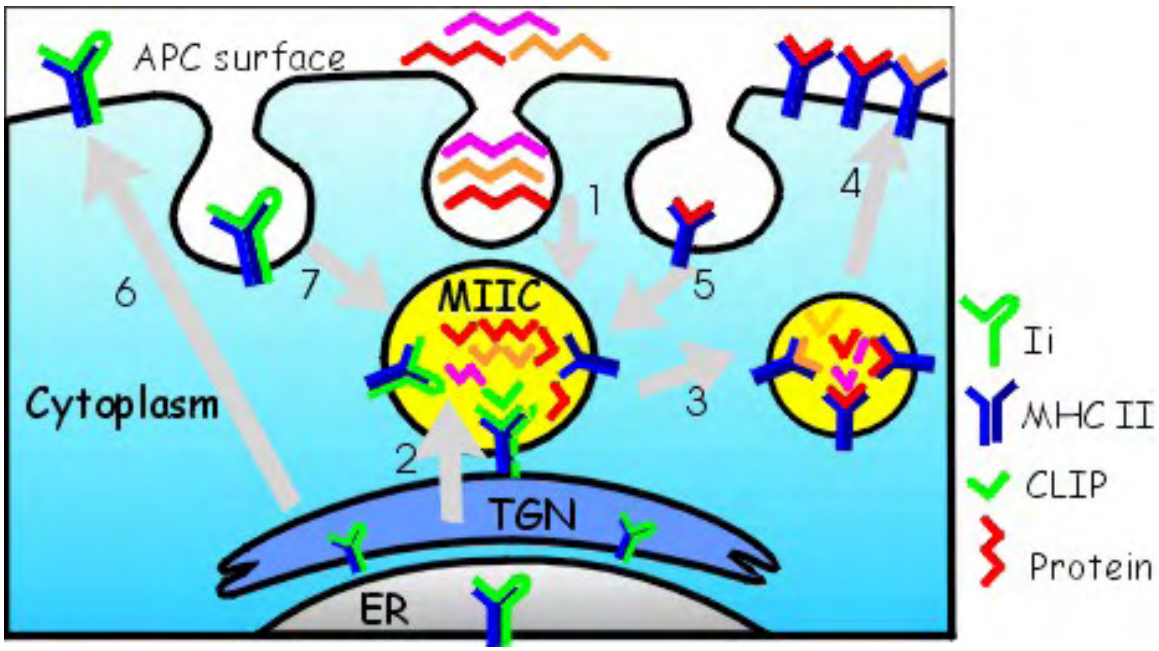


Figure 3. Peptide processing and presentation by MHC II in the presence of Ii
Professional APC endocytose large proteins into the endocytic compartments, where they are processed into smaller peptides (1). Newly synthesized MHC II dimer binds to Ii in the ER and traffics through the trans-golgi network to the endosomal compartments (2). In the late endsomal compartments (MIIC) Ii is degraded and replaced by peptides from exogenous proteins (3). MHC II bound to peptides traffics to the cell surface (4). Cell surface MHC II-peptide complexes can be endocytosed, entering the MIIC again where low affinity peptides may be replaced with higher affinity peptides before MHC II again traffics to the cell surface (5).
Alternatively MHC II-Ii complexes have also been shown to bypass the endosomal compartment and traffic to the cell surface (6). These MHC II-Ii complexes can be endocytosed and enter into the MIIC where Ii is degraded and removed (7). There MHC II can bind exogenously derived peptides and traffic to the cell surface.

E. T cells can be activated by peptides presented via MHC molecules.

T cells have highly polymorphic T cell receptors (TCR) which can bind to and recognize peptides bound to MHC molecules (63). TCR are heterodimeric and can theoretically form 10^{15} different α/β combinations (64). In contrast to MHC molecules which bind a wide array of peptides, TCRs, like antibodies, bind with high specificity to a particular peptide (MHC-peptide complex). T cells can therefore bind to a myriad of antigens including self antigens. To avoid T cell mediated autoimmune disease T cells which have high affinity for self peptide-MHC are eliminated during T cell development, in the thymus, through a process called negative selection. The primary elimination of self reactive T cells is part of a process called 'central tolerance'. Self reactive T cells may still escape negative selection and enter the periphery and for this reason there are safe guards in place to limit the activation of self reactive T cells (65).

To assure that T cells are not activated to self peptide presented via MHC by normal cells, proper priming of naïve T cells requires two signals. The first signal is sent through the TCR when the TCR binds with some affinity to the MHC-peptide complex. The second signal is the costimulatory signal mediated between costimulatory molecules on the APC which interact with a coreceptor on the T cell. One such costimulatory molecule B7.1 (CD80) interacts with CD28 on the T cells (66). If this signal is absent the T cell will become apoptotic or anergic in effect becoming tolerant to its corresponding antigen (67). Since only professional APC such as DC and B cells normally express high levels of costimulatory molecules, professional APC are needed to prime a proper T cell response. These professional APC usually require additional 'danger signals', such as pathogenic factors, inflammatory molecules or factors released by dying cells, in order to differentiate to a state which induces proper T cell activation (68, 69).

After T cells are primed they can undergo further activation when they encounter cells expressing MHC-peptide but no longer require costimulation.

Another safe guard build into the immune system which assures that cytotoxic T cells are not activated against self antigens is that there are two types of T cells which both need to be activated in order to prime an effective T cell response. $CD8^+$ T cells bind to peptide-MHC I complexes and $CD4^+$ T cells bind to peptide-MHC II complexes. Optimal priming of $CD8^+$ T cells requires help from $CD4^+$ T cells. $CD8^+$ and $CD4^+$ T cells have both been shown to play a role in tumor regression (70, 71).

1. CD8 T cells are involved in tumor regression.

Many researchers have focused on the use of tumor specific cytotoxic $CD8^+$ T cells for immunotherapy for two reasons: i) most tumors are positive for class I major histocompatibility molecules (MHC I) and ii) $CD8^+$ T cells directly kill tumor cells. Vaccination with tumor peptides known to activate $CD8^+$ T cells have proven somewhat effective in mouse models (72-75), but are largely ineffective in patients (76). Many mouse cancer vaccines protective effect against tumor is deleted when $CD8^+$ T cells are eliminated (50, 77-79). Therefore $CD8^+$ T cells are important for tumor regression but are not successful as a single agent to eliminate cancer.

2. $CD4^+$ T cells provide help to eliminate tumor.

$CD4^+$ T cells provide "help" to $CD8^+$ T cells by secreting small regulatory protiens (cytokines), such as IFN-γ and IL-2, which bind to corresponding receptors on the T cells stimulating their proliferation and activation. $CD4^+$ T cells also provide help by up regulating CD40, a costimulatory molecule, in the host APC, thus stimulating and 'conditioning' the APC

to differentiate to a state where it can now directly co-stimulate killer T cells (69, 80-82). $CD4^+$ T cells help to induce more prolific $CD8^+$ T cell responses.

Previous studies have shown that although tumor-specific $CD8^+$ T cells can be activated without $CD4^+$ T cell help, long term immunity and optimal $CD8^+$ T cell activation usually requires $CD4^+$ T cell co-activation (83-86). Absence of $CD4^+$ T cells in mouse models leads to loss of antitumor immunity in various vaccine systems including cell-based vaccines, recombinant viral vaccines and recombinant bacterial vaccines (7, 50, 77-79, 87-91). Clinical trials using adoptive transfer of T cells in tumor bearing individuals have also bolstered the importance of $CD4^+$ T cells in tumor regression (92). Many researchers have used $CD8^+$ T cells expanded ex-vivo from patient's TILs with little clinical effect (93). Not until $CD4^+$ T cells were adoptively transferred together with $CD8^+$ T cells, did researchers observe tumor regression in some patients (94).

$CD4^+$ T cells called 'Th1' facilitate tumor regression. Th1 cells secrete cytokines IL-2, IL-3, IFN-γ, IL-12, and TNFα, which activate $CD8^+$ cytotoxic T cells. Th1 cells induce protective antitumor immunity in vivo and aid in inducing $CD8^+$ T cell memory (95, 96). To find out what type of $CD4^+$ T cell was required for tumor regression Sato et al drove T cells to become either Th1 or Th2 (which activate more of an antibody response) and found that their tumor vaccine was more effective when the T cell response was a Th1 type(97). Patients with greater numbers of tumor infiltrating Th1, than Th2 cells have a more favorable prognosis (98, 99). Therefore it may be preferential to induce a Th1 response against tumor as opposed to a Th2 response. We show that, at least in vivo, our tumor vaccines induce a Th1 response (chapter 3, 4).

3. Activated $CD4^+$ T cells lead to better $CD8^+$ T cell activation.

$CD4^+$ T cells are needed to maintain $CD8^+$ T cell effector and memory function as well as survival (100-102). $CD4^+$ T cells are needed to maintain proliferation and viability of adoptively transferred $CD8^+$ T cells in cancer patients (103). Schoenberger and colleagues have shown that $CD8^+$ T cells primed in the absence of CD4 help are not "programmed" and undergo activation-induced cell death when restimulated with antigen (104, 105). Therefore optimal activation of $CD8^+$ T cells requires $CD4^+$ T cell help.

4. $CD4^+$ T cells are required for $CD8^+$ T cell memory.

Immunological memory requires the presence of $CD4^+$ T cells (106, 107). Preclinical studies in mice looking at the importance of $CD4^+$ T cells in treating existing tumor have shown that, while $CD8^+$ T cells, if properly activated ex-vivo, can effectively remove existing tumor without the aid of $CD4^+$ T cells, mice still eventually succumb to later metastasis. Recurrence of primary tumor or metastatic disease that may occur years after removal of primary tumor can only be targeted long term through the use of $CD4^+$ T cells together with $CD8^+$ T cells (108).

5. $CD4^+$ T cells may have tumor specific cytotoxicity.

$CD4^+$ T cells have been termed helper T cells (Th) because, until recently, they have not been shown to directly kill cells but help to activate CTL. More recently some $CD4^+$ T cells have been shown to have antigen specific cytotoxicity (109, 110). Tumor specific cytotoxic $CD4^+$ T cells have been expanded from cancer patients and shown to target malignant but not normal tissue (111-113). Cytotoxic $CD4^+$ T cells have been shown to kill through the same mechanisms as $CD8^+$ T cells, via a perforin-mediated pathway (109). Tumor specific cytotoxic

$CD4^+$ T cells could aid in tumor elimination and would be an added benefit to immunotherapies targeted at activating tumor specific $CD4^+$ T cells.

Given the critical role of Th1 $CD4^+$ T cells we have developed vaccines which have the potential to activate tumor-specific Th1 cells. MHC II^+ Ii^- vaccines have been shown to activate Th1 T cells in mice (114). Chapter 2 describes the development of MHC II^+ Ii^- tumor vaccines and how they may present antigen to activate Th1 cells.

F. MHC II^+ tumor cells directly activate tumor specific $CD4^+$ T cells to multiple novel antigens in the absence of Ii.

Removing Ii changes the antigen processing and presentation of peptides associated with MHC II, driving more presentation of endogenous peptides. Ii has been shown to inhibit presentation of endogenous peptides by MHC II (115, 116). Miller et al proved that MHC II could properly form and traffic to the cell surface in the absence of the Ii chaperone (117). Ostrand-Rosenberg et al hypothesized that tumors expressing MHC II in the absence of Ii, would preferentially present endogenously synthesized antigens thereby facilitating the presentation of a broader range of tumor antigens not otherwise accessible to MHC II in the presence of Ii. This concept has been confirmed by studies showing that in the absence of Ii, MHC II^+ cells more efficiently present endogenous antigens than are presented in the presence of Ii. (118-120). By adding various trafficking signals to a model antigen Qi et al was able to observe presentation of intracellular antigens from individual organelles via MHC II. They found that in the absence of Ii, intracellular antigens from various organelles were presented and activated T cells better than in the presence of Ii (121). Therefore tumor cells expressing MHC II in the absence of Ii present more endogenous antigens, many of which are not presented in the presence of Ii.

In the absence of Ii MHC II presents endogenous antigens. In the absence of Ii MHC II is free to bind peptide in the ER, similar to MHC I, yet MHC II in the absence of Ii does not require elements of the MHC I pathway for antigen processing and presentation. The exogenous antigen processing and presentation pathway of MHC II in the presence of Ii is well characterized, but the endogenous pathway in the absence of Ii is not well characterized. In the absence of Ii, MHC II would be free to bind peptides in the ER, much like MHC I. Therefore Dissanayake et al looked at elements involved in the processing and presentation of antigens via MHC I to see if they were also involved in MHC II antigen processing and presentation in the absence of Ii. Dissanayake et al found that MHC II antigen presentation was not dependent on elements of MHC I presentation such as the proteosome or TAP (122, 123). Therefore MHC II, in the absence of Ii, may not bind to smaller peptides processed by the MHC I pathway, but may bind to ER resident peptides or peptides brought into the ER by transporters other than TAP. Alternatively, MHC II may not need to bind small peptides in the ER, but could bind whole proteins or large peptides that are later chewed back by proteases in the endosome leaving smaller peptides bound to MHC II (124). It is still unclear if MHC II, in the absence of Ii, binds peptides in the ER or later. MHC II dimer is highly unstable when its peptide binding groove is unoccupied suggesting that MHC II binds peptide in the ER. Peptides bound in the ER may, however, be replaced in later compartments by higher affinity peptides that are normally involved in the MHC II antigen presentation pathway.

Even in the absence of Ii MHC II may traffic through the endosomal pathway and load antigen in the endosome. Dissanayake et al further analyzed the effect of classical MHC II antigen processing and presentation elements in the absence of Ii and found that the endosome was still necessary for presentation of endogenous antigens (122). Because Ii is known to

contain an endosomal trafficking signal it was believe that Ii was needed to traffic MHC II through the endosome. Germain et al found that the tail of the MHC II β chain contains a leucine-based motif implicated in transport to the early endosome (125). This supports the findings by Dissanayake et al that the endosome is needed for presentation of endogenous antigens by MHC II in the absence of Ii (122).

Because Ii negative cells express more intracellular peptides via MHC II these peptides must come in contact with MHC II somewhere before the late endosome or MIIC. Ii is normally degraded in the late endosome, if intracellular peptides bound MHC II in the late endosome then there would be no difference in binding with or without Ii. Therefore binding of intracellular peptides must occur somewhere before the late endosome, possibly early endosomes, TGN or the ER. Some endogenous antigens require functional endosomes, therefore endogenous antigens must bind MHC II in the absence of Ii in early endosomal compartments before the MIIC.

Since MHC II in the absence of Ii can bind to antigens from various organelles, these antigens must traffic to where MHC II can bind them. The endosome seems a likely target for delivery of antigens, since MHC II passes through the endosome and it is there where peptides are most weakly associated, allowing for loading of higher affinity peptides. Some have theorized that the process of autophagy may deliver cytosolic antigens to endosomal compartments (126). Dissanayake et al blocked autophagy in MHC II^+ Ii^- cells and showed that it was not involved in presentation of a hen egg lysozyme (HEL) cytosolic model antigen via MHC II in the absence of Ii (122, 123). Yet others have shown that this process is involved in delivery of some intracellular antigens to endosomes (127). Therefore the mechanism of delivering peptides to compartments within the MHC II pathway is still controversial.

HLA-DM, which is also up-regulated by CIITA, does not negatively influence endogenous antigen presentation via MHC II in the absence of Ii. In the absence of Ii HLA-DM may still affect the peptide repertoire by replacing low affinity peptides with higher affinity exogenous peptides. Qi et al looked at the effects of HLA-DM on MHC II antigen presentation in the absence of Ii. They found that even though HLA-DM did aid presentation of exogenous peptides in the absence of Ii, it did not lower the presentation of endogenous HEL peptides in the absence of Ii (128). These MHC II^+ Ii^- tumor cells were equally effective tumor vaccines with or without DM (128). Therefore, in the absence of Ii, HLA-DM does not affect MHC II presentation of endogenous peptides.

Given these finding a tentative antigen processing and presentation pathway is depicted in **figure 4** which describes how novel endogenous antigens are presented via MHC II in the absence of Ii. In the ER, either whole proteins, or small peptides degraded by ER resident proteases can bind to MHC II. Then MHC II traffics through the TGN to the endosome. In the acidic endosome, cathepsins 'chew back' proteins leaving small peptides bound to MHC II and the low pH allows for release and loading of higher affinity antigens brought into the endosome either through autophagy or some, as of yet unidentified, mechanism. Then MHC II-antigen traffics to the cell surface. MHC II^+ Ii^- cells present a wide array of unique endogenous peptides not presented in the presence of Ii. Therefore, $MHCII^+Ii^-$ modified tumor cells could induce a more prolific response to multiple tumor antigens making it harder for tumors to escape detection through down-regulation of a single target antigen. These novel antigens presented via MHC II at the cell surface are unique to MHC II^+ Ii^- cells and can potentially induce high affinity T cells which have not previously encountered these novel antigens.

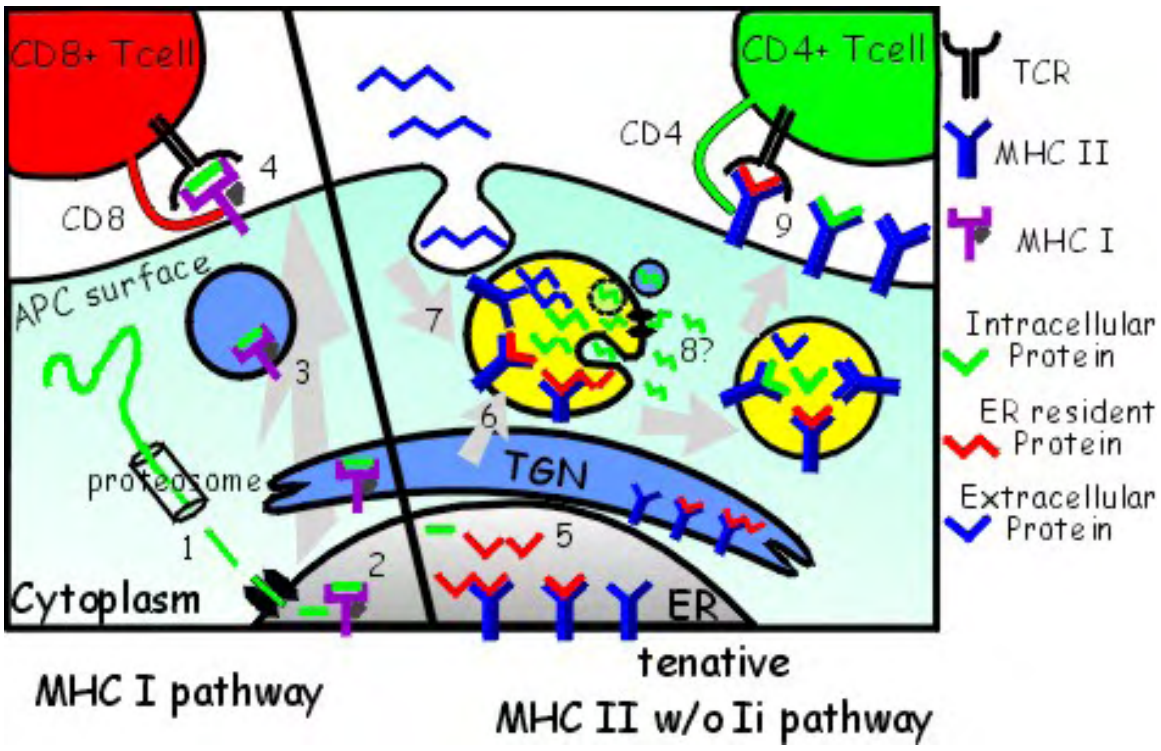


FIGURE 4. Peptide processing and presentation by MHC I compared with that of MHC II in the absence of Ii.

MHC I

Cellular proteins are recycled through ubiquitination and delivered to the proteosome which degrades them into smaller peptides (1). These peptides enter the ER through transporter associated with antigen processing (TAP) and then bind to MHC I (2). MHC I-peptide complexes traffic to the cell surface through the TGN (3). MHC I-peptide binds to T cell receptors on $CD8^+$ T cells activating them (4).

MHC II

In the ER newly formed MHC II dimers are free to bind to ER resident proteins and peptides in the absence of Ii (5). Naked MHC II and/or MHC II bound to small or large peptides traffics through the TGN to the early endosome (6). Extracellular peptides may be endocytosed and enter the endosomal pathway, entering either early or late endosomes (7). Intracellular or endogenous proteins may enter endosomes through some unknown mechanism such as autophagy or through a protein channel or endocytosis (8). In the early endosome large peptides bound to MHC II would be digested into smaller peptides and peptides could be exchanged for higher affinity peptides. MHC II-peptide complexes could traffic to the cell surface and bind to T cell receptors on $CD4^+$ T cells (9).

G. In the absence of Ii novel antigens are presented which may activate high affinity T cells.

Current immunotherapies which attempt to induce an immune response against normally expressed tumor antigens may only activate T cells with low to moderate affinity because high affinity T cells for self antigens are deleted during T cell development. Many shared tumor antigens are self antigens presented in the thymus in the same form as is presented in tumor cells in the periphery. Even tumor antigens not expressed in other tissues of the body and previously believed to be tumor specific are expressed in the thymus (129-131). Some therapies s have successfully activated T cells against tumor antigens in mouse tumor model (132), but few treatments targeting tumor antigens/self antigens have proven clinically effective (76). Because tumors are self cells, tumor immunotherapies attempt to induce tissue specific autoimmunity against tumor cells. Some autoimmune diseases are known to arise because APC present different epitopes (cryptic epitopes) than are presented by thymic epithelial cells during negative T cell selection (133). This difference in antigen repertoire has been shown to be due to differences in antigen processing between different cell types. In one case studied, DC processed different insulin peptides than did thymic epithelial cells, leading to diabetes (134). Therefore, changes in antigen processing may lead to activation of high affinity T cells not deleted during negative selection. Novel antigens presented in the absence of Ii may induce high affinity T cells to tumor antigens much like cryptic self epitopes may activate T cells to self antigens in some autoimmune diseases.

Tumors may induce T cell tolerance thereby escaping elimination by the immune system (135-137). Wide spread release of tumor antigens may lead to T cell tolerance to those antigens and it may be necessary to overcome tolerance with $CD4^{+}$T cell help (138). Tumor antigens may

be presented in the thymus and therefore delete antigen specific T cells during negative selection. Chronic expression of tumor antigens over time may also tolerize T cells in the periphery (65). Therefore the longer a tumor can evade removal by the immune system, the more tolerized the immune system will become to that tumor. In cancer patients with advanced disease, high affinity T cells may no longer exist with specificity to tumor antigens which are normally expressed. It may however be possible to activate $CD4^+$ T cells, which have not been tolerized, to novel tumor antigens because these antigens have not been present during tumorigenesis.

In the absence of Ii novel endogenous antigens are presented by MHC II molecules. Muntasell et al genetically engineered MHC II expressing cells with and without Ii to characterize the peptides presented by MHC II in the presence or absence of Ii by isolating and analyzing those peptides by mass spectroscopy (139). Their findings support the model antigen findings (116, 119-121) that show improved endogenous antigen binding in the absence of Ii. Most antigens presented by MHC II in the absence of Ii were of intracellular origins and came from the ER, cytosol, mitochondria, or nucleus. Interestingly they also found that 9 of the 10 cytosolic peptides identified from MHC II^+ Ii^- DM^- cells corresponded to N- or C-terminal epitopes of the source proteins. This may give a clue as to how peptides are loaded onto MHC II in the absence of Ii. Together, the Muntasell et al. and Qi et al. (121, 139) studies are compelling evidence supporting our hypothesis that a different repertoire of peptides is presented in the absence of Ii.

In the absence of Ii, MHC II^+ APCs activate different T cells than are activated in the presence of Ii. To study the effect of Ii on the MHC II antigen repertoire Bodmer et al probed spleen cells from wild type and Ii knock out mice with a large panel of antigen-specific T cell hybridomas (118). They found that Ii blocked the presentation of many antigens but a few

antigens were presented only in the presence of Ii. They also found that Ii^+ mice were not tolerant to epitopes expressed in Ii^- cells, suggesting that Ii^+ mice do not present the same antigens as Ii^- mice. The Muntasell and Bodmer findings support my hypothesis that novel antigens are presented by MHC II in the absence of Ii, and these novel antigens presented in the absence of Ii may activate T cells not activated by Ii^+ APC.

Novel self antigens have the potential to activate high affinity T cells inducing a more prolific response than induced by lower affinity self antigens. Because MHC II and Ii are controlled by the same 'master switch', CIITA, they are always expressed together, even during T cell development where negative selection against T cells with high affinity to self antigens occurs. If Ii independent antigens are not presented in the thymus during T cell differentiation, then high affinity T cells for these antigens would not be deleted. T cells with high affinity exist for non self antigens such as viral antigens, not present during T cell development in the thymus. This may be why viral antigens induce more prolific T cell responses where up to 10% of all circulating T cells are specific to that antigen, as opposed to most responses to tumor/self antigens where only 1-2% of circulating T cells may be antigen-specific. Therefore tumor cells expressing MHC II in the absence of Ii may present novel tumor antigens which could activate high affinity T cells in vivo much like truly novel antigens such as viral antigens. This hypothesis is explored in chapter 4 of this thesis.

H. Multiple tumor antigens must be targeted to eliminate tumors which are heterogeneous.

Tumor immunotherapies which activate T cells to multiple tumor antigens may be needed to remove existing tumors which are highly heterogeneous in nature. Tumors are genetically

unstable which causes them to be very heterogenous in their gene expression (140-142). Loss of a particularly immunogenic antigen or loss of the particular MHC allele which presents that antigen may allow a particular tumor cell to escape immune detection (143, 144). Therefore, therapies which target only one antigen may be limited due to the heterogeneous and mutable nature of tumors. Because therapies which target activation of T cells to one antigen have not yielded therapeutic results many researchers are trying to develop therapies which activate T cells to multiple tumor antigens (144). I hypothesize that MHC II^+ Ii^- tumor cells will present multiple endogenous tumor antigens, which can potentially activate multiple tumor specific T cells.

I. Controversy exists as to whether MHC II is functional without Ii.

Some mouse MHC II alleles are more Ii dependent than others. Initial findings that MHC II was deficient in the absence of Ii were from I-A^b knockout mice where the following phenotype was observed. Ii-knock out I-A^b mice expressed lower levels of MHC II on the cell surface of DC. Crossing these B6 (I-A^b) Ii knock outs onto other genetic backgrounds showed that other mouse MHC II alleles were less dependent on Ii and had less pronounced 'defects'. It was found that mice expressing other MHC II alleles such as I-A^k and I-A^d expressed more cell surface MHC II in the absence of Ii than those with the I-A^b allele (145). Furthermore, DC from mouse Ii-knock out with the I-A^k allele matured and presented stable MHC II on their cell surface, unlike the DC from mice with the I-A^b allele (146). One of the extreme phenotypes of the B6 (I-A^b) Ii-knock out mice was their deficiency of $CD4^+$ T cells, since MHC II expression on the cell surface of thymic epithelial cells is involved in the development of these cells (147). Ii-knock out mice with the I-A^k and I-A^d alleles had functional $CD4^+$ T cells (145, 148). I-A^k and

I-A^d presented self peptide and were able to activate T cells (149). So even though cell surface levels of MHC II in mice with the I-A^k and I-A^d alleles were still somewhat lower than in Ii^+ mice of the same allele, nonetheless these I-A^k and I-A^d MHC II molecules were still expressed and functional.

Much of the belief that MHC II binds more weakly to antigen or is 'naked' in the absence of Ii is again based on the findings in B6 (I-A^b) Ii knock out mice. Neither A^k nor A^d in the absence of Ii forms 'floppy dimers' (145, 150). Therefore, the formation of unstable floppy dimers by the B6 strain of mice may be an exception and not representative of most alleles.

Much of the confusion as to whether MHC II is functional in the absence of Ii is based on early researchers' definition of what is functional MHC II expression. Most researchers consider that MHC II is not functional in a cell if that cell cannot process and present exogenous antigens. Ii^- cells have been shown to be less effective at processing and presenting some exogenous antigens (115). Later papers characterizing the broader effects of MHC II expression of antigens in the absence of Ii showed that endogenous antigen expression was not effected by the absence of Ii (145). Others showed an enhanced expression of endogenous antigens from various organelles (121). Therefore in the absence of Ii exogenous antigens may not be presented as well via MHC II but endogenous antigens are presented.

When we look at expression of endogenous antigens in Ii knocked out I-A^k and I-A^d mouse models, MHC II^+ Ii^- cells are not impaired and may even express a wider array of antigens than cells with Ii (121, 139, 145). Kenty et al studying expression of exogenous antigens showed that Ii^- cells (I-A^d), when pulsed with whole exogenous proteins, presented 3 of 4 exogenous antigens expressed with Ii^+ cells. Looking at presentation of endogenous antigens Kenty et al found that Ii^- cells presented intracellular peptides derived from whole proteins better

than cells with Ii (145). Studies looking at previously identified endogenous antigens presented via MHC II are limited and do not represent the broad repertoire of peptides that may be presented in the absence of Ii. All the endogenous antigens known to be presented by MHC II have been identified in Ii^+ cells; Ii^- cells may present many endogenous peptides not presented in the presence of Ii. Findings from Bodmer et al showed that presentation of many endogenous antigens was blocked by Ii, whereas some antigens were Ii indifferent (presented both with and without Ii) or Ii dependent (118). MHC II^+ Ii^- human cell lines while unable to process and present whole protein antigens added exogenously, were able to present endogenously synthesized influenza peptides (119, 120). These MHC II^+ Ii^- cells lost endogenous antigen presentation if transfected with Ii (119, 120). Revisiting previous findings stating that MHC II is not functional in the absence of Ii we find that MHC II does not functionally present some exogenous antigens but does present endogenous antigens in the absence of Ii.

Although few mouse MHC II alleles are truly Ii dependent, no human alleles have shown such sensitivity. Stable expression of cell surface MHC II in the absence of Ii has been shown in transfected human fibroblasts (151-153). These studies have shown that MHC II is functional in the absence of Ii and can present endogenous antigens activating allele-restricted, antigen-specific T cells proving that MHC II is functional in presenting endogenous antigens in the absence of Ii. Some researchers theorized that these transfected cell lines were Ii independent due to cell-specific factors. This theory was an attempt to explain why mouse Ii-knock out splenocytes expressed lower levels of cell surface MHC II, whereas human fibroblasts transfected with MHC II and not Ii express normal levels of stable cell surface MHC II. Kenty et al's findings led them to believe that this was not a cell type dependent phenomenon, but did find that MHC II^+ Ii^- cells from the Ii knock out mice if left in culture expressed higher MHC II levels

on the cell surface over time(145). They theorized that the phenotype of cultured cells changed to explain the discrepancy. Cultured MHC II^+ Ii^- cells expressed somewhat lower cell surface levels of MHC II than Ii^+ cells. In contrast, many genetically modified human cell lines expressing human MHC II in the absence of Ii have shown no difference in cell surface levels of MHC II compared with Ii^+ lines. Because the human cell lines do not behave phenotypically like mouse cells they are either less Ii dependent or not Ii dependent at all. Whether there are human MHC II alleles that are more Ii dependent than others, is not known. However, all alleles tested in our lab (DR1, 4, 15, 7) give stable cell surface expression equivalent to those found in Ii^+ cells ((154) and unpublished data by Thompson, Bosch, and Srivastava).

Our vaccine strategy relies on the fact that MHC II, in the absence of Ii, does not present the same repertoire of peptides presented by MHC II in the presence of Ii. Therefore, T cells activated by the novel peptides presented by MHC II in the absence of Ii may not have been tolerized.

J. Summary

Patients who develop metastatic disease fail to mount a sufficient immune response to eliminate tumor. The immune system can recognize tumor antigens and specifically target tumor metastasis. Due to mechanisms of tolerance tumors may escape immune surveillance. Tumor immune therapies which activate a prolific immune response may lead to tumor regression. Because of the importance of $CD4^+$ T cells in tumor regression I have developed tumor cells which express MHC II and costimulatory molecules for use as tumor vaccines. In order to activate more high affinity $CD4^+$ T cells to endogenous tumor antigens I have expressed MHC II without Ii. Chapter 2 will describe the development of an MHC II expression vector used to

create the MHC II^+ vaccine cells. Chapter 3 describes studies that test the ability of MHC II to express a known tumor antigen with and without Ii. Chapter 4 describes experiments that show the effect of Ii on antigen presentation and what T cells are activated with and without Ii. Experiments done in chapter 4 support my hypothesis that MHC II expressed in the absence of Ii can activate a stronger immune response against tumor than can MHC II in the presence of Ii.

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Chapter 2

Invariant chain negative human tumor cells transduced with a novel tricistronic retroviral vector express stable MHC Class II

Excerpts from: Dissanayake SK, Thompson JA, Bosch JJ, Clements VK, Chen PW, Ksander BR, Ostrand-Rosenberg S. *Activation of tumor-specific CD4+ T lymphocytes by major histocompatibility complex class II tumor cell vaccines: a novel cell-based immunotherapy*. Cancer Res. 2004 64:1867-1874.

ABSTRACT

Mouse tumor cells transfected with syngeneic MHC class II and costimulatory molecule genes are therapeutic vaccines in mice, provided they do not co-express the class II-associated Invariant chain (Ii). We previously demonstrated that the vaccine cells present tumor peptides via the endogenous antigen presentation pathway to activate $CD4^+$ and $CD8^+$ T cells. Because of their efficacy in mice, we are translating this vaccine strategy for clinical use. To obtain MHC class $II^+CD80^+Ii^-$ human tumor cells we developed retroviruses encoding HLA-DR and CD80. The HLA-DR virus encodes the DRα and DRβ0101 chains using an internal ribosomal entry site to coordinate expression. SUM159PT mammary carcinoma and Mel 202 ocular melanoma cells transduced with the retroviruses (/DRB1/CD80) express high levels of DRB0101 and CD80 on the cell surface in the absence of Ii as shown by immuno-fluorescence and Western analysis. Therefore it is possible to express MHC II on human tumor cells in the absence of Ii. These MHC II^+ Ii^- $CD80^+$ tumor cells may function as tumor vaccines to reduce or prevent metastatic disease and increase the survival of cancer patients.

INTRODUCTION

A key goal of cancer vaccine development is to generate therapeutic reagents that provide protection against development and outgrowth of metastatic tumor cells. Since metastatic disease for many tumors appears at varied intervals after diagnosis of primary tumor, the most effective vaccines will provide long-term immune memory. We (1, 2) and others (3-5) have focused on the critical role of $CD4^+$ T cells in cancer vaccines, because these cells, in conjunction with $CD8^+$ T lymphocytes, are likely to provide maximal anti-tumor immunity with long-term immunological memory.

To better activate tumor-specific $CD4^+$ T cells we have designed cell-based vaccines that facilitate the presentation of MHC class II-restricted tumor peptides to responding $CD4^+$ T cells (2). We have reasoned that tumor cells present a variety of MHC-restricted peptides that are potential tumor antigens, and that if they constitutively express MHC class I molecules and are transduced with syngeneic MHC class II and costimulatory molecules, they could function as antigen presenting cells (APC) for MHC class I and class II-restricted tumor peptides. This approach is appealing for several reasons: 1) Identification of specific tumor antigen epitopes is not required; 2) Multiple class I and class II-restricted epitopes will be presented concurrently; and 3) T cells may be activated to novel tumor epitopes not presented by professional APC.

To test our approach, cell-based vaccines were generated from mouse SaI sarcoma, B16 melanoma, and 4T1 mammary carcinoma by transfection with syngeneic MHC class II alpha and beta genes and with costimulatory molecule (CD80) genes (2, 6). These tumor lines were selected because they do not constitutively express the MHC class II accessory molecule, Invariant chain (Ii), which blocks presentation of endogenous antigen and interferes with vaccine efficacy (7, 8). These vaccines provide potent prophylactic immunity (1) and mediate tumor

rejection in mice with established primary, solid tumors (9). The vaccines are also effective in reducing experimental metastatic disease (10), and they reduce spontaneous metastatic tumor burden and significantly prolong survival of mice with spontaneous metastatic mammary carcinoma (11, 12). In vitro and in vivo studies demonstrated that the genetically modified vaccine cells enhance anti-tumor immunity because they directly present endogenously synthesized tumor encoded epitopes to reactive $CD4^+$ T lymphocytes, and thereby activate $CD8^+$ T cells and provided long-term immunity (7, 8, 13).

Because of its therapeutic efficacy in mice, we are translating this vaccine approach for the treatment of human cancers. Our experimental design is to express syngeneic MHC class II and costimulatory molecules in established human tumor cell lines that constitutively express MHC class I molecules and do not constitutively express Ii. To achieve this goal we are using retroviral transduction to express HLA-DR and CD80 molecules in two human tumor lines, an ocular melanoma (Mel 202) and a mammary carcinoma (SUM159PT). The resulting HLA-DRB0101-transduced cells stably express high plasma membrane levels of functional HLA-DRB0101, as measured by immunofluorescence. If human cells replicate what has been found in mouse models, the MHC II^+ Ii^- $CD80^+$ cells developed in this study may serve as powerful tumor vaccines in patients.

MATERIALS AND METHODS

Construction of retroviral vectors. pLNCX2/DR1 construct: DRA cDNA in the RSV.5 vector (14) was PCR amplified including 5' *NheI* and 3'*XhoI* restriction sites. DRA 5' primer: TGTCGCTAGCATGGCCATAAGTGGAGT; DRA 3' primer: ACTGCTCGAGTTACAGAGGCCCCCTGCGTT. The PCR product was cloned into the

pCR2.1-TA vector (Invitrogen, Carlsbad, California), excised with *NheI* and *EcoRI*, and inserted into the multiple cloning site (MCS)-A of *NheI* and *EcoRI* digested pIRES plasmid (Clontech, Palo Alto, CA). DRB0101 in the RSV.5 vector (14) was PCR amplified including 5' *XmaI* and 3' *NotI* sites and subcloned into the 5' *XmaI* and 3' *NotI* sites of the MCS-B of the pIRES vector. DRB0101 5' primer: AGTACCCGGGATGGTGTGTCTGAAGCTC. DRB01013' primer: TAGTGCGGCCGCTCAGCTCAGGAATCCTGTTG. PCR conditions for both DRA and DRB0101 amplifications were: denature at 94 C for 2 min., denature at 94 C for 1 min., anneal at 60.9 C or 62.9^{o} (DRA and DRB0101, respectively) for 1 min, extend at 72 C for 3 min (High Fidelity Taq, Roche, Basel Switzerland); repeat the last three steps 30 times, extend at 72 C for 7 min. The resulting construct is pIRES/DR1 (**Figure 1A**).

The pLNCX2 retroviral vector (Clontech) was modified to include a linker containing an *AvrII* site in the MCS. To make the linker, equimolar amounts of the oligonucleotides (5' GATCTCGAGCTCCTAGGAATTGTTTGGCCGAGGC-3' and 3' AGCTCGAGGATCCTTAACAAACCGGCTCCGCCGG5') were mixed, heated at 95° C for 5 min, and then incubated at 22° C for 1 hr. The resulting linker was ligated to *BglII* and *NotI* digested pLNCX2. The resulting construct is pLNCX2/*Avr II*.

The DRA-IRES-DRB0101 fragment of the pIRES/DR1 was digested with *NheI* and *NotI* and gel purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA), and then ligated to *AvrII* and *NotI* digested pLNCX2/*Avr II*. The final MHC class II construct is pLNCX2/DR1 (Figure 1A).

pLHCX/CD80 (Hph) construct: pLHCX (hygromycin resistance; Clontech) was modified to include a 5'*BamHI* site and a 3'HindIII site by inserting an oligonucleotide linker between the *HindIII* and *ClaI* sites of the MCS. The original *HindIII* in the vector was deleted

by insertion of the linker. *XhoI, HpaI, AvrII*, and *NotI* restriction sites were included in the linker for future cloning purposes. The linker sequence was:

L1 5'-AGCTGCTCGAGTTAACGGATCCTAGGAAGCTTGCGGCCGCAT-3'

L2 5'-CGATGCGGCCGCAAGCTTCCTAGGATCCGTTAACTCGAGC-3'

Human CD80 was excised from the pREP10/B7.1 vector with *BamHI* and *HindIII* and inserted into the modified pLHCX vector using the *BamHI* and *HindIII* sites (Figure 1B).

pLPCX/CD80 (puro) construct: The CD80 gene was excised from pREP10/B7.1 by digestion with *BglII* and *HindIII* and ligated into pLPCX digested with *BamHI* and *HindIII*. The *BamHI* and *BglII* sites were deleted during this process (Figure 1C).

Cells. Media for all cell lines contained 1% gentamicin, 1% penicillin/streptomycin (all from BioSource, Rockville, MD) and 2mM Glutamax (BRL/Life Sciences, Grand Island NY). All cells and T cell activation assays were cultured at 37^{o}C in 5% $CO_{2.}$ SUM159PT was obtained from the Michigan Breast Cell/Tissue Bank (www.cancer.med.umich.edu/breast_cell/umbnkdb.htm) and was maintained in Hams F-12 medium with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, Utah), 1μg/ml hydrocortisone, and 5μg/ml insulin (both from Sigma, St. Louis MO). Mel 202 (16) was grown in RPMI 1640 medium (BioSource, Rockville, MD) with 10% FCS, 0.01M HEPES (Invitrogen, Grand Island NY), and 5×10^{-5} M β-mercaptoethanol (J.T. Baker Inc, Phillipsburg, NJ). Transductants were grown in the same medium as their parental cells, supplemented with G418 (Sigma), puromycin (Clontech, Palo Alto, CA) or hygromycin (Calbiochem, San Diego, CA) (see **Table 1** for dosages), depending on their transgenes. Sweig and Jurkat cells were obtained

from the American Type Culture Collection and were maintained in IMDM medium (BioSource, Rockville, MD) supplemented with 10% fetal clone I (FBP) (Hyclone).

Retrovirus production. 293T cells (obtained from the Harvard Gene Therapy Institute) were plated in a 6 cm dish at $9x10^5$ cells/4 ml 293T medium [DMEM (BioSource, Rockville, MD) 1% gentamicin, 1% penicillin/streptomycin, 1% Glutamax, and 10% heat–inactivated FCS] and cultured at 37°C. Twenty to 24 hrs later the growth medium was replaced with 4ml of 37°C IMDM medium containing 25mM Hepes (BioSource, Rockville, MD)1% Glutamax, and 10% heat-inactivated FCS. Three hours later the 293T cells were transfected with pLNCX2/DR0101, pLHCX/CD80 or pLPCX/CD80 plasmids (8 μg) plus pMD.MLV gag.pol (6 μg) and pMD.G (2 μg) using $CaPO_4$ (17). Twelve to 16 hrs post transfection, medium was replaced with 293T growth medium containing 10mM Hepes. Virus was collected 48 hrs later and either used immediately or stored at –80ºC.

Retroviral transduction. $1.2\text{-}3.0\times10^5$ tumor cells were plated in 6 cm dishes in 293T medium. Approximately 16 hours after plating, when cells were in log phase, 1 ml of a 1:2 dilution (293T medium containing 10 mM Hepes) of viral supernatant containing 4 μg/ml polybrene (Sigma) was added and the mixture was incubated at 37°C for 5-6 hours. Transduced cells were then washed twice with excess PBS, and maintained in growth medium for two days before adding G418, puromycin, and/or hygromycin.

Antibodies, reagents, and immunofluorescence. Mabs (HLA-DR-FITC and CD80-PE), streptavidin-PE, FITC-isotype, and PE-isotype controls were purchased from BD Pharmingen

(San Diego, CA). Biotinylated HLA-DR0101 was purchased from One Lambda Inc (Canoga Park, CA). Rat anti-mouse IgG-FITC was purchased from ICN (Costa Mesa, CA), and CD4-FITC, CD8-FITC, and anti-human IgG-FITC were purchased from Miltenyi Biotech (Auburn, CA). Human IgG-FITC was purchased from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A, B, C), L243 (pan anti- HLA-DR), 28.14.8 (anti-H-2L^d, D^b), and PIN1 (anti-Ii) were purified on Protein A or Protein G affinity columns as previously described (1). Tumor cells were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8 and Ig) or fixed and stained for internal markers (Ii) by direct or indirect immunofluorescence as previously described (1, 18).

HLA-DR nomenclature. The HLA-DR gene used in these studies was sequenced and identified as HLA-DRB0101, and is abbreviated as “DR1" in the names of the transductants.

RESULTS

Construction of retroviruses encoding HLA-DRα plus HLA-DRβ, and CD80. To generate human tumor cells expressing high levels of MHC class II molecules, retroviruses encoding HLA-DRα plus HLA-DRβ genes have been generated. The HLA-DRB0101 allele was selected because it is one of the more common alleles in the Caucasian population and is a frequently used restriction element (21, 22). A novel bicistronic retroviral vector that drives coordinate expression of approximately equimolar amounts of HLA-DRα and HLA-DRβ was developed using the pLNCX2(neo) retroviral backbone. DRα and DRβ0101 cDNAs (14) were cloned upstream and downstream, respectively, of the internal ribosomal entry site (IRES) of the vector pIRES. The DRA-pIRES-DRB segment was then excised from the pIRES vector and ligated

into the pLNCX plasmid to yield the pLNCX/DRβ1 plasmid (**Figure 1A**). This construct will produce a single chain mRNA driven by the CMV promoter in which DRα is translated by a CAP dependent mechanism and DRβ is translated via the IRES in a CAP-independent manner.

Because of the critical role of costimulatory molecules in activation of naive T cells (23), we have also generated retroviral plasmids encoding human CD80 (hCD80). The hCD80 gene was excised from the pREP10/B7.1 plasmid and ligated into the retroviral vector pLHCX(hph) or pLPCX(puro) to form the pLHCX/CD80 (**Figure 1B**) or pLPCX/CD80 (**Figure 1C**) plasmids, respectively.

All retroviral plasmids were packaged in 293T cells, and supernatants containing infectious retroviruses were harvested and used to transduce target tumor cells.

Transduced human tumor cells express cell surface HLA-DRB0101 and CD80. The human ocular melanoma cell line, Mel 202, and the mammary carcinoma cell line, SUM159PT, were transduced with different combinations of the pLNCX2/DR1, pLHCX/CD80 and pLPCX/CD80 retroviruses. The resulting transductants are shown in **Table 1**. SUM159PT and Mel 202 tumors were chosen because they do not constitutively express MHC class II molecules and hence should not express Ii, which we have previously shown inhibits presentation of MHC class II-restricted endogenous antigens (7, 8). To assess the magnitude and stability of transgene expression, transductants were tested by immunofluorescence and flow cytometry one week after being placed on drug selection (see **Table 1** for drug selection conditions for each transductant line) and intermittently for six months thereafter. As shown in **Figure 2**, Mel 202 and SUM159PT transductants express high levels of cell surface HLA-DR (L243 mAb) and CD80 (CD80-PE mAb) as measured at six months after transduction. The parental lines and

transductants were also stained for MHC class I molecules (W6/32 mAb). All lines showed strong class I expression, with transductants displaying levels roughly comparable to their parental lines (data not shown).

To ascertain that the MHC class II expression is allele-specific, SUM/DR1 and SUM/DR1/CD80 cells were stained for cell surface expression of HLA-DR1 using the HLA-DR1 specific mAb. As shown in **Figure 3**, pLNCX2/DR1-transduced SUM cells express high levels of DR1, and only stain at background levels with an irrelevant HLA-DR2-specific mAb. Therefore, SUM/DR1/CD80, SUM/DR1, SUM/CD80, Mel 202/DR1, Mel 202/CD80, and Mel 202/DR1/CD80 transductants express high levels of the transduced HLA-DR and/or CD80 genes as measured by antibody reactivity and immunofluorescence.

SUM159PT and Mel 202 cells do not express invariant chain. Because co-expression of Ii inhibits endogenous antigen presentation by MHC class II vaccine cells (7, 8), SUM159PT and Mel 202 cells were tested to ascertain that they do not express Ii. Cells were permeabilized, stained with the Ii-specific mAb PIN-1, and analyzed by flow cytometry. As shown in **Figure 4A**, neither tumor line contains Ii, while the human B cell line, Sweig, which constitutively expresses Ii is strongly positive. To further confirm the absence of Ii, detergent extracts of SUM159PT, Mel 202, Ii-positive Sweig, and Ii-negative Jurkat cells were electrophoresced by SDS-PAGE and analyzed by western blotting for Ii expression. As shown in **Figure 4B**, neither SUM159PT, Mel 202, Mel 202/DR1/CD80 nor SUM/DR1/CD80 cells contain detectable Ii, although Ii expression is inducible in SUM159PT cells by a 48 hr treatment with IFNγ. Therefore, SUM159PT and Mel 202 tumor cells do not constitutively express Ii, so Ii will not be

present in the transduced vaccine cells to inhibit binding and presentation of endogenously synthesized peptides.

DISCUSSION

During the past approximately ten years animal studies and some clinical trials have indicated that the use of genetically engineered tumor cells as vaccines may have therapeutic efficacy for the treatment of cancer (25-27). Parallel studies have recognized the critical role played by $CD4^+$ T cells in orchestrating the host immune response against cancer, and have developed methods to activate $CD4^+$ T cells
(2-5, 28-30). Because $CD4^+$ T cells play a central role in enhancing anti-tumor immunity, our laboratory has focused on facilitating the activation of these cells. We have hypothesized that tumor cells that constitutively express MHC class I molecules, do not contain Ii, and are genetically modified to express syngeneic MHC class II molecules and costimulatory molecules will function as antigen presenting cells for *endogenously* synthesized MHC class I and class II-restricted tumor antigen epitopes. Furthermore, if used as immunogens in tumor-bearing individuals, such cells will serve as "vaccines" to activate tumor-specific $CD4^+$ and $CD8^+$ T lymphocytes that will facilitate tumor regression (2, 6). Because the efficacy of these vaccines has been demonstrated in multiple mouse tumor models (9, 11, 12, 31, 32), the goal of this study was to translate this strategy for clinical use.

Activation of tumor-specific $CD4^+$ T cells by the cell-based vaccines is based on the supposition that the MHC class II molecules of the vaccine cells bind peptides synthesized within the tumor cells and directly present these peptides to $CD4^+$ T lymphocytes. This mode of presentation is different from that of professional APC, which typically bind peptides derived from endocytosed exogenously synthesized antigens (33). This fundamental difference is

attributable to the absence of the MHC class II-associated accessory molecule, Ii, in the vaccine cells. If APC express Ii, Ii binds to newly synthesized MHC class II molecules, thereby preventing the binding of endogenously derived peptides and favoring the binding of exogenously synthesized peptides (34). However, in the absence of Ii, MHC class II molecules bind peptides derived from endogenously synthesized antigens (7). Since the MHC class II and Ii genes are coordinately regulated and coordinately induced by IFNγ (35), professional APC and tumor cells that constitutively express MHC class II genes and/or are induced by IFNγ are unlikely to be APC for endogenously synthesized tumor antigens. Studies with Ii^+ and Ii^- MHC class II^+ tumor cells support this concept, and demonstrate that the most efficacious vaccines are MHC class II^+Ii^- (7, 8, 18).

Early studies suggested that expression of MHC class II molecules without co-expression of Ii produces reduced levels of class II molecules that are improperly conformed and unable to function as antigen presentation elements (36-38). More recent studies have demonstrated that the Ii dependency of MHC class II molecules is allele specific (39, 40), and that many MHC class II alleles do not require Ii expression for stability or antigen presentation function (41). The studies reported here demonstrating efficient antigen presentation by MHC class II^+Ii^- tumor cell vaccines add HLA-DR0101 to the list of MHC class II alleles whose expression and function is independent of Ii co-expression.

In addition to the absence of Ii for maximal vaccine efficacy, extensive mouse and human studies show the requirement for costimulation for optimal T cell activation (reviewed in (23)). This study has shown that the CD80 costimulatory molecule is stably expressed in tumor vaccine cells. Many studies have also shown that CD80 expression facilitates tumor rejection (42-44).

A significant technical obstacle in generating the MHC class II cell-based vaccines was to routinely achieve high level expression of the desired MHC class II alleles in human tumor cells. Since many human tumor cells and cell lines can be problematic to maintain in culture, standard transfection and electroporation techniques did not result in reproducible class II expression (S. Dissanayake and J. Bosch, unpublished results). In contrast, transduction using a bicistronic retrovirus encoding the DRα and DRβ chain genes separated by an IRES routinely yielded high level HLA-DR expression in a high proportion of transductants. The efficiency of the current retroviruses appears to be due to the placement of the DRα and DRβ genes flanking the IRES, since a previous study using a retroviral construct encoding pig DQα and DQβ genes run off of separate promoters and without an IRES, produced only low level DQ-expressing cells (49). It is likely the IRES construct will be universally useful, since similar retroviruses encoding other HLA-DR alleles also reproducibly yield high level MHC class II expression in additional human tumor lines (51).

MHC II^+ Ii^- $CD80^+$ human tumor cell lines have been shown to activate a prolific $CD4^+$ T cell response in vitro (51). The potency of the MHC class II vaccines for activating $CD4^+$ T cells to tumor encoded antigens suggests that these vaccines may have therapeutic efficacy for cancer patients. For example, the cell-based vaccines could be administered in vivo to patients with disseminated metastatic disease. Alternatively, they could be used ex vivo to activate patients' T cells for subsequent adoptive transfer. In either case, these vaccines provide a novel and potent approach for activating tumor-specific $CD4^+$ T cells and merit further clinical development and testing.

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Table 1. Tumor cell vaccines (transductants) used in these studies.

Cell Line	HLA-DRB0101	CD80	Drug Selection
SUM/DR1	+	---	G418[b]
SUM/CD80	---	+	HPH[c]
SUM/DR1/CD80	+	+	G418 + Puro
Mel 202/DR1	+	---	G418
Mel 202/CD80	---	+	HPH[e]
Mel 202/DR1/CD80	+	+	G418 + Puro

[b] 600 μg/ml
[c] 200 μg/ml
[d] 0.2 μg/ml
[e] 75 μg/ml

Figure 1

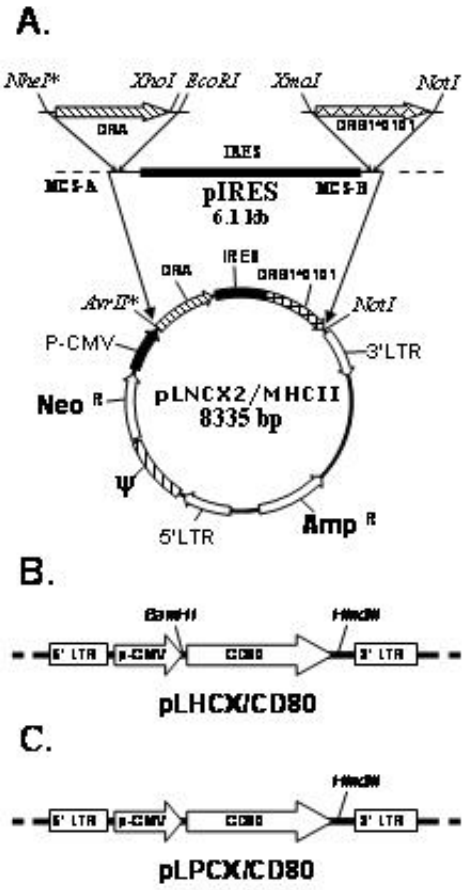

Figure 1. Retroviral constructs made and used in these studies. (A) The pLNCX2/MHC II construct contains the DRA and DRB0101 cDNAs flanking an IRES and under the control of the CMV promoter, and contains the G418 resistance gene. (B) and (C) The pLHCX/CD80 and pLPCX/CD80 constructs encode the human CD80 gene and contain the hygromycin or puromycin resistance genes, respectively. (D) The pLPCX/TT construct encodes the tetanus toxin fragment C gene and contains the puromycin resistance gene.

Figure 2

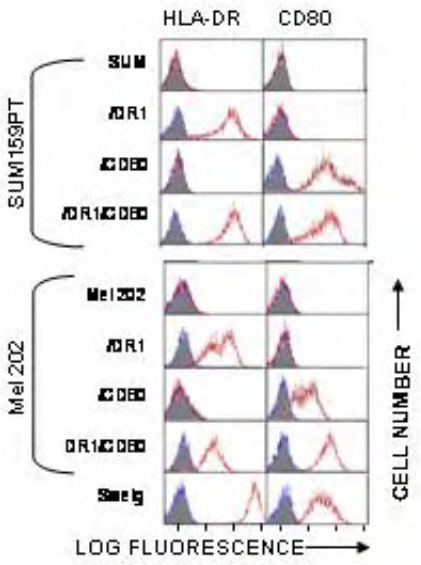

Figure 2. SUM159PT and Mel 202 transductants express MHC class II HLA-DR and CD80 on the cell surface and TT internally. Live transductants were stained by direct immunofluorescence for plasma membrane HLA-DR (L243-FITC) or CD80 (CD80-PE). Fixed cells were stained by indirect immunofluorescence for internal TT (TT mAb plus fluorescent conjugate). Gray peaks denote staining with fluorescent conjugate alone or isotype control; white peaks represent staining with directly coupled primary antibody or primary antibody plus fluorescent conjugate. These data are from one of three to five independent experiments.

Figure 3

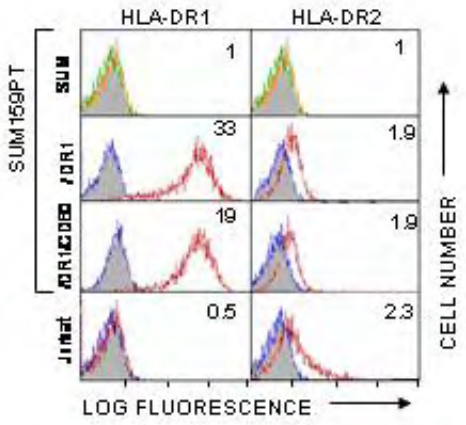

Figure 3. SUM/DR1 and SUM/DR1/CD80 transductants express HLA-DR1 at the cell surface. Live cells were stained by indirect immunofluorescence for plasma membrane HLA-DR1 (mAb HLA-DR1 biotin) or with an irrelevant Ab (HLA-DR2-biotin) plus an avidin-FITC conjugate. Jurkat is a $DR1^-$ cell line. Gray peaks denote staining with fluorescent conjugate without primary antibody. White peaks represent staining with primary antibody plus fluorescent conjugate. Numbers in the upper right hand corner of each profile are the mean channel fluorescence for the antibody stained peak. These data are from one of two to five independent experiments.

Figure 4

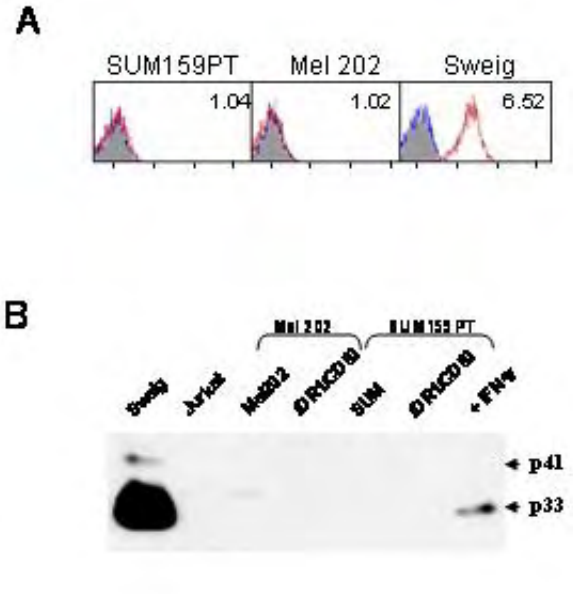

Figure 4. Sum159PT and Mel 202 cells do not express Ii chain. (A) Fixed cells were stained by indirect immunofluorescence for Ii (mAb PIN1.1; white peaks) or with fluorescent conjugate alone (gray peaks). These data are representative of three independent experiments. (B) Uninduced or IFNγ-treated (+ IFNγ) cells were detergent lysed, electrophoresed on 10% SDS-PAGE gels under reducing conditions, and transferred to nitrocellulose. Blots were stained for Ii with the mAb PIN1.1. Sweig and Jurkat cells are Ii^+ and Ii^- cell lines, respectively. These data are from one of two to three independent experiments.

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Chapter 3

Tumor Cells Transduced with the MHC Class II Transactivator and CD80 Activate Tumor-Specific CD4$^+$ T Cells Whether or Not They Are Silenced for Invariant Chain

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Tumor Cells Transduced with the MHC Class II Transactivator and CD80 Activate Tumor-Specific $CD4^+$ T Cells Whether or Not They Are Silenced for Invariant Chain

by

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Key words: Cancer vaccine, HER2/neu, invariant chain, MHC class II transactivator, MHC class II antigen presentation, CD4+ T lymphocytes

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ABSTRACT

The specificity and potency of the immune system make immunotherapy a potential strategy for the treatment of cancer. To exploit this potential we have developed cell-based cancer vaccines consisting of tumor cells expressing syngeneic MHC class II and costimulatory molecules. The vaccines mediate tumor regression in mice and activate human $CD4^+$ T cells in vitro. Previous vaccines were generated by transducing MHC II^- tumor cells with a single HLA-DR allele. Since expression of multiple MHC II alleles would facilitate presentation of a broader repertoire of tumor antigens, we have now transduced tumor cells with the MHC class II transactivator (CIITA), a regulatory gene that coordinately increases expression of all MHC II alleles. Previous studies in mice indicated that co-expression of the MHC II accessory molecule, Invariant chain, inhibited presentation of endogenously synthesized tumor antigens and reduced vaccine efficacy. To determine if Ii expression affects presentation of MHC class II-restricted endogenously synthesized tumor antigens in human tumor cells, HLA-DR^- MCF10 breast cancer cells were transduced with the CIITA, CD80 costimulatory molecule gene, and with or without small interfering RNAs (siRNA) specific for Ii. Ii expression is silenced >95% in CIITA/CD80/siRNA transductants, down-regulation of Ii does not affect HLA-DR expression or stability, and Ii^+ and Ii^- transductants activate human $CD4^+$ T cells to DRB1*0701-restricted HER2/neu epitopes. Therefore, tumor cells transduced with the CIITA, CD80, and with or without Ii siRNA present endogenously synthesized tumor antigens and are potential vaccines for activating tumor-specific $CD4^+$ T cells.

INTRODUCTION

Immunotherapy is a potential approach for the treatment and/or prevention of cancer because of its specificity, sensitivity, potency, and long-term memory. T lymphocytes, the cellular arm of the immune response, are particularly promising because they have the capability of localizing to tumor sites and directly killing tumor cells. Because of these characteristics, vaccines and/or immunotherapy may facilitate the destruction of existing disseminated metastatic tumor cells and protect individuals against the recurrence of primary tumors and/or the outgrowth of latent metastatic cells (1, 2).

T cells that are cytotoxic for tumor cells are typically $CD8^+$ T lymphocytes, and optimal activation of these cells usually requires co-activation of $CD4^+$ T helper lymphocytes (3, 4). $CD4^+$ T lymphocytes are also required for generating $CD8^+$ T memory cells (5-7). Because of these critical roles for $CD4^+$ T cells, we are developing cancer vaccines that specifically target the activation of $CD4^+$ T cells, while concurrently activating cytotoxic $CD8^+$ T lymphocytes.

$CD4^+$ T lymphocytes are activated to peptide antigen that is presented by major histocompatibility complex (MHC) class II molecules. Since MHC II molecule expression is usually limited to professional antigen presenting cells (APC), immunity to most pathogens requires that professional APC acquire antigen from exogenous sources. To facilitate the presentation of endocytosed antigen, professional APC contain the MHC class II associated accessory molecule, invariant (Ii) chain. Ii hinders the presentation of endogenously synthesized peptides and favors the presentation of antigen acquired by endocytosis. It mediates this effect by binding to newly synthesized MHC class II molecules in the endoplasmic reticulum (ER) and preventing them from acquiring peptides of endogenously synthesized molecules. The Ii chain also contains trafficking signals which guide newly synthesized MHC II molecules to the

endocytic pathway where Ii protein is degraded and peptides derived from endocytosed proteins bind (reviewed by (8, 9)). In professional APC, MHC class II and Ii molecules are coordinately regulated at the transcriptional level by the MHC class II transcriptional activator (CIITA), a master regulatory gene that controls expression of all MHC II alleles (10-13). This coordinate regulation assures that professional APC efficiently present antigenic peptides acquired from extracellular sources.

Ii has been considered essential for MHC II function. It's requirement is supported by the finding that Ii-knockout mice have very low levels of MHC class II molecules, and $CD4^+$ T cell activation and function is minimal, or have dysfunctional MHC II molecules (14-18). In contrast, some Ii negative non-professional APC when transfected or transduced with MHC class II genes present antigen and activate $CD4^+$ T cells (16, 19-22), suggesting that MHC class II molecules can be fully functional in the absence of Ii.

Based on the assumption that co-expression of Ii blocks endogenous antigen presentation, we have produced cancer vaccines by transducing MHC II^- tumor cells with syngeneic MHC II and costimulatory molecule genes. The vaccines mediate tumor regression in mice and activate tumor-specific $CD4^+$ T cells (20, 23-26). Activated $CD4^+$ T cells in both mouse and human systems are specific for antigens encoded by the vaccine cells. These vaccines have been produced by transducing MHC class II^- tumor cells with a single HLA-DR allele. Since expression of multiple MHC II alleles may facilitate presentation of a broader repertoire of tumor antigens, we have now transduced tumor cells with the CIITA. To determine if Ii co-expression affects T cell activation to endogenous antigen, we have introduced small interfering RNAs (siRNA) specific for Ii into human tumor cells transduced with the CIITA and CD80 costimulatory molecule genes. The transductants efficiently activate human $CD4^+$ T cells to

HER2/neu tumor antigen epitopes, suggesting that this strategy may be useful for vaccine design. Properly conformed and functional MHC II heterodimers are present in transductants with or without Ii siRNA, indicating that Ii is not essential for HLA-DR function. Surprisingly, transductants with or without the Ii siRNA are equally efficient at activating $CD4^+$ T cells, indicating that in this system, Ii does not impair endogenous antigen presentation.

MATERIALS AND METHODS

Cells. SUM159PT, Jurkat, Sweig, 293T, and PBMC were handled as described (20). The human breast cancer line MCF10CA1 (hereafter called MCF10) and it's non-malignant counterpart, MCF10A were cultured in MCF10 medium (DMEM/Hams F12 (1:1), 5% heat-inactivated FCS, 0.029M Na bicarbonate,10mM HEPES (27)) or MCF10A medium (MCF10 medium supplemented with 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin (all from Sigma), and 20 ng/ml epidermal growth factor (Gibco/BRL)), respectively. MCF10 transductants were supplemented with puromycin (0.3 μg/ml; Clontech, Palo Alto, CA), or hygromycin (150 μg/ml; Calbiochem, San Diego, CA). The OMM2.3 human ocular melanoma line (28) was grown in RPMI with 10% heat-inactivated FCS, and 5×10^{-5} M β-mercaptoethanol. All cell lines and procedures were approved by the Institutional Review Boards of the participating institutions.

SiRNA. Complementary sequences in the coding region of the human Ii gene (GenBank NID NM_004355) were identified using the Ambion siRNA target finder search engine (Ambion, Inc. Austin, TX). Sequences with no homology to other known human mRNAs were chosen at random from the 3' to 5' end of the Ii mRNA. SiRNAs were produced by in vitro transcription with T7 RNA polymerase (29) using the Ambion Silencer™ siRNA Construction

Kit and were made homologous to sequences 4, 8, 16, 24, and 50 (double adenine regions found by the Ambion target finder search engine). SiRNAs were transfected into 293T/CIITA cells (20) using 40ng siRNA. SiRNAs producing 4-50 fold decreases in Ii expression were identified and oligonucleotide siRNA expression cassettes were prepared by MWG Biotech (High Point, NC) and inserted into the pSIREN Retro-Q vector (Clontech, Palo Alto, CA) according to the manufacturer's directions (Clontech). The foreward (f) and reverse (r) primers were annealed and ligated to the linearized pSIREN-RetroQ vector with *BamHI* and *EcoRI* 'sticky ends'.

Retroviral Constructs, Transductions, and Drug Selection. The human CIITA gene was cloned from pcDNA1-amp/tagCIITA (30) into Litmus28 (New England Biolabs, Beverly, MA) using *XbaI* and *EcoRI*, then cloned into a modified pLNCX retroviral vector (pLNXC2(AvrII); neo resistance) (20) using *Bgl II* and *AvrII*.

The HLA-DRB1*0701 cDNA (in RSV.5 vector) (31) contained two point mutations: a guanine instead of an adenine at base 13, and an adenine instead of a thymine at base 191. These errors were corrected using Splicing by Overlapping Extensions (SOEing) (32) using the following four primers in successive SOEing reactions: Primer1-AGTACCCGGGATGGTGTGTCTGAAGCTCCCTG, Primer 2-AGCGCACGAACTCCTCCTGGTTATAGAA, Primer 3-TTCTATAACCAGGAGGAGTTCGTGCGCT, Primer 4-TAGTGCGGCCGCTCAGCTCAGGAATCCTGTTG. Reaction 1: 10 pM RSV.5/DRB1*0701 template and 0.5 μM of primer 1 and 2: cycle at 95° C 2min, then 30 cycles of 95° C 30sec, 60.2 ° C 30sec, 72° C 1 min. then 72° C for 10min. Reaction 2: 10 pM RSV.5/DR1*0701 template and 0.5 μM primer1 and 2: same as PCR1 but annealing temperature was 62.3. Reaction 3: 10 pM of product from reactions 1 and 2 were mixed with primers 1 and 4 and incubated at 95° C x

2min, followed by 5 rounds of 95° C x 30sec, followed by 60.2 C x 30sec, followed by 72° C x 1min. Then 5 rounds more of the same with annealing temperature at 62.3° C, followed by 23 rounds of the same at 64.8° C, followed by 72° C x 10min. All reactions used 2 units of PFU turbo polymerase (La Jolla, CA) according to the manufacturer's specifications. These primers added the *XmaI* and *NotI* restriction sites on the 5' and 3' ends of the cDNA, respectively. The corrected sequence was confirmed by sequencing of both strands.

Using the same restriction sites as for cloning of pLNCX2/HLA-DR1 the HLA-DRB1*0701 or DRB1*0401 genes were cloned into the downstream site of the pIRES/DRA0101 vector containing the HLA-DRA*0101 gene in the upstream site. The DRA0101-IRES-DRB1 section was excised from the resulting vector and cloned into the retroviral vector pLNCX2 (*AvrII*). The pLHCX/CD80 retroviral construct, retrovirus production, and transductions were previously described (20).

Transduced cells were selected as follows: CD80 transductants: 150μg/ml hygromycin; DR4, DR7 and CIITA transductants: 300 μg/ml G418; siRNA transductants: 0.3μg/ml puromycin. If 2-3 weeks of drug selection did not yield homogeneous populations of transgene expressing cells, the transductants were sorted by magnetic bead selection (Miltenyi, Auburn, CA) according to the manufacturer's directions.

Peptides, Antibodies, Reagents, and Immunofluorescence. HER2/neu peptide 98-114 (RLRIVRGTQLFEDNYAL) and peptide 776-790 (GVGSPYVSRLLGICL) (33, 34) were synthesized at the University of Maryland Biopolymer Laboratory. Monoclonal antibodies [mAbs; HLA-DR-FITC and CD80-PE], streptavidin-PE, FITC-isotype, and PE-isotype controls were from BD PharMingen (San Diego, CA). Biotinylated HLA-DR1 mAb (BIH0126) was from One Lambda, Inc. (Canoga Park, CA); HLA-DQ-PE and HLA-DP-FITC from Chemicon

(Temicula, CA); rat anti-mouse IgG-FITC from ICN (Costa Mesa, CA); c-neu (Ab-2) from Oncogene (Cambridge MA); CD4-FITC, CD8-FITC and anti-human IgG-FITC from Miltenyi Biotech; and human IgG-FITC from Cappel (West Chester, PA). Culture supernatants of hybridomas W6/32 (pan HLA-A, B, C), L243 (pan anti- HLA-DR), PIN1.1 (anti-Ii) and 28.14.8 (anti-H-$2D^bL^d$) were prepared and tumor cells and PBMC were stained for cell surface markers (MHC class I, class II, CD80, CD4, CD8, and immunoglobulin) or fixed and stained for Ii as described (20).

Western Blots. Ii Western blots were performed as described (20). Blots for MHC II were done as for Ii with the following modifications: Cell lysates were loaded onto SDS-PAGE gels using non-reducing loading dye (0.2% SDS, 20% glycerol, 1.25M Tris pH 6.8 and 0.4mg/ml bromophenol blue). Half of each sample was boiled for 5 min immediately before loading. Blocking buffer was 2% bovine serum in TBST. Membranes were incubated with undiluted supernatant from hybridoma L243 and the last wash was done for one hour.

T cell priming. PBMC from healthy donors (2×10^7 cells/4 ml/well) were cultured in PBMC medium (Iscove's modified Dulbecco's medium, 10% FCS, 1% penicillin, 1% streptomycin (BioSource, Rockville, MD), 2mM Glutamax (BRL/Life Sciences, Grand Island, NY)) with 2μg/ml of HER2 p98 or p776 in 6-well tissue culture plates at 37°C and 5% CO_2 for 5 days. Non-adherent cells were harvested, washed twice with PBMC medium and re-plated in 24 well plates with 20 units/ml of recombinant human IL-2 (R&D Systems, Minneapolis, MN) at $1x10^6$ cells/2 ml/well. HER2-activated non-adherent cells were harvested seven days later, live cells isolated using Histopaque-1077, cultured 1-5 days without exogenous IL-2, and used the following day. For some experiments, after the incubation with IL-2, non-adherent PBMC were cultured at 1×10^7/4ml/well with $8x10^6$, 50 Gy-irradiated SUM/DR7/CD80 cells for five days,

washed and cultured as above with IL-2 for seven days, and washed and rested for one day before use.

Alternatively, PBMC were obtained from HER2/neu immunized patients with Stage III or IV breast, ovarian, or non-small cell lung cancer participating in a U. of Washington FDA-approved Phase I trial (35). Patients were immunized intradermally once a month for 6 months to the same regional draining lymph node site with three different peptides derived from HER2/neu and admixed with 100μg GM-CSF. PBMC were collected one month after the last immunization and cryoperserved. For ex vivo boost, PBMC, were thawed at 37^{o}C, washed twice, and resuspended at 3×10^{6} cells/ml in X-VIVO media (10% human AB serum, 2mM L-glutamine, 20mM HEPES buffer, and 10mM acetylcysteine solution (USP). The cells were stimulated with 10mg/ml of HER-2/neu peptides (p98, p776, or p98+p776) and incubated at 37^{o}C in 5% CO_2 for 12 days. On days 4/5 and 8 10U/ml of recombinant human IL-2 (Chiron Corporation, Emeryville, CA) and 10ng/ml of recombinant human IL-12 (R&D System) were added. On day 12 the cells were harvested, washed, counted, tested by flow cytometry and ELIspot, and resuspended at 1×10^{6}/ml in fresh media containing 1×10^{5}/ml of antiCD3/CD28-coated beads to a final concentration of 1 or 10 bead(s) per T cell. Between days 14 and 23 the cell concentration was evaluated every two to three days and the cells were diluted to 0.5-1×10^{6}/ml with fresh media as needed. On days 15, 18, 20, and 22 IL-2 was added to a final concentration of 30U/ml, and on day 25 the expanded cells were harvested, washed, counted and evaluated by flow cytometry and ELIspot (36).

Antigen Presentation Assays. Antigen presentation assays and T cell depletions were performed as described (20) using Miltenyi human CD4 and CD8 beads with the following modifications: Stimulator cells were used at 2.5×10^{4} cells/well. MCF10-derived and MCF10A

stimulator cells were not irradiated; all other stimulators were 50 Gy-irradiated. Antibody blocking experiments included 20 μg/ml L243 (anti-HLA-DR), W6-32 (anti-class I MHC) or 28.14.8. For exogenous HER2 peptide presentation, assays were as for endogenous antigen presentation, except soluble HER2 peptide p98 or p776 was included at 2ug/ml.

HLA-DR Nomenclature and Genotypes. Normal donor PBMC are A24, A29, B44, B35, DR7, DR11, and DRβ3,4. SUM159PT cells are A2, A24, B5, and B15, DR4, and DR13. OMM2.3 cells are A11, A29, B7, and B52. MCF10 and MCF10A cells are A33, B55, B22, DR7, DR4. HLA genotypes were determined by PCR typing and are referred to by their short-hand form (e.g. HLA-DR7 is DRB1*0701).

Statistical Analyses. Means, standard deviations, and statistical significance as measured by Student's T test were calculated using Excel v2002.

RESULTS

Small Interfering RNA Down-Regulates Ii. The human breast cancer epithelial cell line MCF10 (27), which does not constitutively express MHC class II or Ii molecules, was transduced with a retrovirus encoding the human CIITA gene (**figure 1A**), and selected by magnetic bead sorting for MHC class II expression. CIITA transduced MCF10 (MCF10/CIITA) cells are MHC class II (HLA-DR, DP, and DQ) and Ii positive as shown by immunofluorescence (**figure 1B**).

Retroviruses expressing siRNAs for Ii were constructed to down-regulate Ii in MCF10/CIITA cells. Four sequences in the human Ii gene, starting with AA and having low GC content were selected (#4, #38, #16, and #50). Double stranded RNA molecules of these sequences were prepared and were transiently transfected into 293T cells that had previously

been transduced with the CIITA retrovirus. Sequences 4 and 50, but not 38 or 16, down-regulated Ii by 4 to 50 fold (data not shown). To obtain stable transductants, sequence #4 was cloned into the pSIREN vector with two different termination signals giving vectors 4.1 and 4.2, with 4.2 containing additional sequence following the six thymidine. Sequence #50 was not used because it contained a four thymidine repeat that is a stop of transcription for the U6 pol III promotor. Additional sequences starting with AA and having low GC content were selected adjacent to sequence #50 and these were inserted into pSIREN (sequences #48, #53, and #54). An additional sequence, #32, was also randomly selected and inserted into pSIREN. Sequences were inserted using the forward and reverse primers shown in **figure 1C**. Retroviruses containing these six siRNAs were prepared and used to transduce MCF10, MCF10/CIITA, and CD80 expressing cells (MCF10/siRNA, MCF10/CIITA/siRNA, MCF10/CIITA/CD80/siRNA cells). Transduced cells were analyzed by flow cytometry three days after transduction. Lines containing siRNAs #32, #53 and #48 showed a marked down-regulation of Ii but not complete loss, whereas cells containing siRNAs #4.1, #4.2 and #54 had minimal down regulation of Ii (data not shown).

Western analyses for Ii (mAb PIN1.1) were performed three days after transduction to confirm that Ii expression was down-regulated. The predominant form of Ii in MCF10/CIITA cells is p35, with a smaller amount of p33 (**figure 2A**). The p35 isoform, which is translated via an alternative translation initiation site, is normally the less abundant isoform of Ii (37). Others have noted an increase in p35 in tumors (38). The p35 and p33 isoforms are both down-regulated >95% in the siRNAs #53, #48 or #32 transductants, and there is a slight down-regulation in siRNA #4.1. SiRNAs #4.2 and #54 do not affect Ii expression. Interestingly, at three days post-transduction all of the down-regulated cell lines contain a 23 kDa band that corresponds to an Ii

degradation product. To ascertain if p23 persists, MCF10/CIITA/siRNA #32 and #53 were cultured in selective media for three weeks and subsequently tested by western analysis. Neither full length Ii nor the Ii degradation product is present three weeks after selection (**figure 2B**). Similar results were obtained with MCF10/CD80/CIITA/siRNA cells (data not shown).

To determine if siRNA-mediated down-regulation of Ii affected cell surface expression of MHC class II molecules, the transductants were analyzed by immunofluorescence three weeks after siRNA transduction. HLA-DR expression is the same in siRNA and non-siRNA transductants (**figure 2C**, upper panels). Ii is absent in MCF10/CD80/CIITA/siRNA 53, and reduced >95% in lines #32 and #48 (lower panels). Therefore, down-regulation of Ii by siRNA eliminates Ii expression without affecting MHC class II expression.

MCF10/CIITA/CD80 Cells Down-regulated for Ii Contain Stable MHC Class II Heterodimers. Formation of stable αβ heterdimers that dissociate upon boiling is a hallmark of correctly conformed MHC class II molecules (39). To determine if the siRNA transductants have properly conformed MHC II molecules, lysates of > 3 week drug selected cells were either boiled or not boiled prior to western blotting with the L243 mAb, which is specific for MHC II alpha-beta dimers. Non-boiled samples contain bands migrating at ~55Kda that correspond to stable MHC class II αβ heterodimers (**figure 2D**). Therefore, tumor cells transduced with the CIITA and Ii siRNA express properly conformed MHC class II molecules in the absence of Ii.

MCF10/CIITA/CD80/siRNA Cells Present Endogenous HER2/neu Peptides and Activate $CD4^+$ T Cells. To assess if MHC class II is functional in the absence of Ii, MCF10/CD80/CIITA/siRNA 32 cells, which are down-regulated for Ii >99% were used as antigen presenting cells for the activation of peripheral blood mononuclear cells (PBMC) to

tumor-encoded epitopes. HER2/neu, a growth factor receptor that is over-expressed by many tumors, was used as the tumor antigen because 1) MCF10 cells constitutively over-express HER2/neu (**Figure 3A**); 2) HER2/neu contains two HLA-DR7-restricted peptides (p98 and p776) (33, 34); and 3) MCF10/CD80/CIITA cells express DR7. To control for HLA-DR specificity, ocular melanoma OMM2.3 and breast cancer SUM159PT were transduced with CD80 and DR7 or DR4 retroviruses (**figure 3B**). OMM2.3 and SUM159PT cells express HER2/neu, do not express Ii, and the respective transductants express CD80 and DR4 or DR7 (**figure 3C**).

To determine if the CIITA/siRNA cells present endogenously synthesized tumor peptides, HER2/neu peptide p98 and/or p776-activated T cells were co-cultured with transduced tumor cells and IFNγ production was measured. Soluble peptide p98 or p776 was added to some wells to determine if the transductants preferentially present peptides from exogenous sources. MCF10/CIITA/CD80/siRNA 32 and MCF10/CIITA/CD80 present peptides p98 and p776 from endogenously synthesized HER2/neu (**Figure 4A, 4B**). Peptide p776 is presented equally well by MCF10/CIITA/CD80/siRNA 32 and MCF10/CIITA/CD80, while MCF10/CIITA/CD80 cells are slightly better presenters of p98 ($p<0.05$). Both MCF10 cell lines present exogenously pulsed HER2/neu peptides. Exogenous peptide presentation is not significantly better than presentation of endogenous peptide and is not affected by Ii expression. HER2/neu presentation is DR7-restricted since cells lacking DR7 (MCF10/CD80, OMM2.3/DR4/CD80, and SUM159/CD80) do not induce significant IFNγ release. T cell activation is limited to MHC restricted antigen and there is no activation to allogeneic MHC antigens since neither MCF10/CD80 nor SUM159/CD80 cells induce significant IFNγ release. Therefore, down-regulation of Ii by siRNA slightly diminishes presentation of peptide p98, but does not

significantly affect presentation of p776, suggesting that presentation of some epitopes may be Ii-dependent, while presentation of others is independent of Ii. In either case, removal of Ii does not render MHC class II molecules unable to present antigen, indicating that MHC class II antigens are functional in the absence of Ii.

To rule out that PBMC were responding to secreted HER2/neu that is subsequently endocytosed, PBMC were mixed with supernatants from MCF10/CIITA/CD80/siRNA 32 cells. PBMC were pulsed with p98 as a positive control. Peptide-pulsed PBMC produced IFNγ; however, supernatant-pulsed cells did not (data not shown). Therefore, MCF10/CIITA/CD80/siRNA 32 cells are presenting endogenously synthesized molecules.

Peptides p98 and p776 also activate $CD8^+$ T cells, suggesting that they may contain nested MHC class I epitopes (33, 34). The HER2/neu activated T cells share DR7 with the transduced MCF10 cells, and DR7 and A24 with SUM159PT/DR7/CD80 cells. No MHC class I alleles are common between p98 and p776-activated PBMC and transduced MCF10 cells. Since peptides p98 and p776 are presented by both DR4 and DR7, there is the potential for the activation of both $CD4^+$ and $CD8^+$ T cells by SUM159PT/DR7/CD80, but not by MCF10/CIITA/CD80/siRNA 32 cells. To identify which T cells are activated, PBMC were primed with p98 and p776, and subsequently incubated with vaccine cells in the presence of antibodies to MHC class I and/or MHC class II. Antibodies to MHC class II block antigen presentation by MCF10/CIITA/CD80/siRNA-32 and MCF/CIITA/CD80, while antibodies to both MHC I and II block antigen presentation by SUM159/DR7/CD80 cells (**figure 4C)**. To confirm the activation of $CD4^+$ and $CD8^+$ T cells, PBMC were primed with p776 and depleted for $CD4^+$ or $CD8^+$ T cells, prior to activation by MCF10 or SUM159PT transductants. Depletion of $CD4^+$ T cells completely eliminates T cell activation by both MCF10/CIITA/CD80/32 and

SUM/DR7/CD80 cells. Depletion of $CD8^+$ T cells significantly reduces T cell activation by SUM159PT transductants, and has a smaller effect on MCF10/CIITA/CD80/32-induced T cell activation (**figures 4D,E**). This latter effect is probably non-specific since MCF10/CIITA/CD80/32 cells do not share MHC class I alleles with the PBMC. Similar results were obtained with p98-primed PBMC (data not shown).

MCF10/CIITA/32 cells were also included in this experiment (**Figure 4D**) to determine if co-expression of CD80 enhances boosting of HER2/neu-specific $CD4^+$ T cells. In agreement with earlier findings (20), CD80-expressing transductants are better stimulators. Therefore, the transductants activate both $CD4^+$ and $CD8^+$ T cells if they share common alleles with the responding PBMC, and co-expression of CD80 enhances activation.

Non-Malignant Cells Do Not Activate T Cells

A potential problem with cell-based vaccines is that they will activate T cells against non-malignant cells due to cross-reactivity with normal, self antigens. To determine if the MHC II vaccines induce reactivity against non-malignant cells, PBMC were activated with HER2/neu p776 peptide and tested on MCF10 cells and their non-malignant counterpart, MCF10A cells. As measured by flow cytometry, MCF10A cells express HER2/neu, although at slightly lower levels than MCF10 cells (**Figure 5A,** compare to figure 3A). Unlike MCF10 cells, MCF10A cells do not express MHC II molecules; however, they are inducible for MHC II if incubated for 48 hours with 1000 units/ml of rIFNγ. As seen in **Figure 5B**, neither untreated, nor IFNγ-treated MCF10A cells activate T cells. Therefore, tumor-specific T cells are activated by MHC II^+CD80^+ tumor-cell-based vaccines, and not by non-malignant cells of the same tissue origin.

DISCUSSION

A goal of tumor immunotherapy is to activate T lymphocytes to tumor-encoded antigens. Although some tumor peptides have been identified, there are many that are unknown, and it is unclear how diverse an immune response is needed to eradicate tumor cells in vivo. The vaccines (transductants) described here circumvent the identification of tumor antigens and potentially present a diversity of immunogenic peptides. The vaccines were designed to preferentially present endogenously synthesized MHC II-restricted epitopes (1). In previous studies we made vaccines by transducing MHC class II alleles into Ii negative tumor cell (23, 24, 40, 41). We now demonstrate that immunogenic cells can also be made by transducing tumor cells with the CIITA with or without Ii.

Our vaccine design was based on the hypothesis that co-expression of Ii would inhibit the presentation of endogenously synthesized tumor peptides, an hypothesis supported by our own earlier work and extensive work of others in non-tumor systems (8, 9). Recent mass spectroscopy studies (42) provide direct biochemical support for this hypothesis and also provide an explanation for the lack of inhibition of Ii for HER2/neu peptides p98 and p776. These investigators demonstrated that the MHC II molecules of MHC II^+Ii^- cells contain peptides presented by MHC II^+Ii^+ cells plus additional novel peptides which are not presented by MHC II^+Ii^+ cells (42). Since p98 and p776 were originally identified as epitopes presented by MHC II^+Ii^+ professional APC (33, 34) they are most likely in the category of epitopes that are presented by both Ii^+ and Ii^- APC. However, the findings of (42) make it likely that the MHC II^+Ii^- transductants also present novel tumor antigen epitopes that are not presented by professional APC. In this fashion, Ii RNAi vaccines may activate a more diverse repertoire of

tumor-specific $CD4^+$ T cells than professional APC, and may activate T cells that have not previously been tolerized by the tumor.

Clinical studies also support an inhibitory role for Ii. Chamuleau and colleagues have shown that AML patients in complete remission whose HLA-DR^+ myeloid leukemic blasts have low levels of the MHC class II-associated Ii peptide (CLIP), a degradation product of Ii, have a significantly better clinical prognosis than patients whose blasts are DR^+CLIP^+ (43). Similar to AML blasts of progressor patients, DM-deficient mice also have DR^+CLIP^+ APC which are inefficient presenters of endogenously synthesized molecules (44). Preferential expression of the Ii p35 isoform is also associated with increased malignancy in chronic lymphocytic leukemia, and this effect has been attributed to reduced presentation of endogenously synthesized tumor antigens (38). Expression of CLIP is also associated with polarization towards a Type 2 CD4 (Th2) response (45, 46), which may favor tumor progression (47).

Although early studies suggested that Ii expression was essential for MHC class II function (14, 15, 17), there are now many reports demonstrating that MHC II alleles are properly conformed and functional in the absence of Ii (19, 20, 25, 48, 49). The present report extends this conclusion and demonstrates that peptide affinity for MHC II is not affected by Ii, because peptide binding to surface MHC II molecules is similar for Ii^+ and Ii^- cells. Therefore, although Ii may be required during development for expression of some mouse MHC II alleles, most MHC II alleles are stable and functional in the absence of Ii.

We envision that the vaccine strategy described here will be used to generate MHC II allele-specific vaccines from established cell lines. We propose a “cocktail” approach in which a patient will be treated with a mixture of multiple cell lines expressing MHC class I and II molecules matched to their genotype. Following HLA typing, a patient's “semi-customized

cocktail" would be prepared from stocks of frozen transduced cells. This approach depends on the existence of shared tumor antigens and eliminates the need for autologous tumor cells, making it feasible to treat most patients. Although retroviruses could be used to induce MHC II and CD80 moleucles, alternative techniques that are less controversial would be preferable.

This vaccine strategy has the potential to activate T cells to self antigens that are also expressed on non-malignant cells. Autoimmunity has not been observed in the three mouse tumor systems studied in vivo (Ostrand-Rosenberg and Clements, unpublished), and the absence of reactivity with the non-malignant breast line MCF10A suggests that autoimmunity against normal cells will also not be a problem in patients. In addition, a $DR4^{+}DR7^{+}$ patient with advanced metastatic ocular melanoma has been treated with irradiated OMM2.3/CD80/DR4 and OMM2.3/CD80/DR7 vaccines, and no autoimmune or other complications were noted (S. Slavin, personal communication).

Vaccines consisting of tumor cells transduced with the CIITA and Ii siRNAs have several potential advantages over previous vaccines in which MHC II non-expressing tumor cells were transduced with individual MHC II alleles. Vaccines made by transducing single HLA-DR alleles are limited to presenting tumor antigen epitopes restricted by the transduced allele(s). In contrast, CIITA transduced vaccine cells express multiple HLA-DR alleles, as well as DP and DQ alleles, and hence have the potential to present a much broader repertoire of tumor antigen epitopes. The CIITA/siRNA vaccines also differ from the previous MHC II vaccines in that expression of the CIITA up-regulates accessory molecules such as HLA-DM. Although our previous studies have not demonstrated that HLA-DM expression facilitates vaccine efficacy (50), studies by others have shown that HLA-DM expression stabilizes MHC II in the absence of Ii, aids MHC II traffic, and helps edit the MHC II peptide repertoire (42).

The CIITA/Ii siRNA strategy also expands the choice of tumor cells which could be used to generate vaccines to include tumors that constitutively express MHC II or are inducible for MHC II by treatment with IFNγ. Tumor cells that constitutively co-express MHC II and costimulatory molecules, such as some leukemias (43), are particularly attractive targets for Ii siRNA therapy since creating a vaccine would only require down-regulating Ii via RNAi. This approach is supported by studies in which Ii was down-regulated in MHC II positive Ii positive mouse tumor cells by anti-sense RNA (51-55). Mice immunized with the Ii anti-sense down-regulated tumor cells were protected against later challenge with wild type tumor. Since siRNA is more effective in down-regulating Ii than anti-sense RNA, Ii siRNA vaccines may have more therapeutic efficacy than Ii anti-sense vaccines. Therefore, tumor cells expressing the CIITA and costimulatory moelcules may be useful reagents, and concommitant down-regulation of Ii via RNAi may further improve vaccine efficacy and protect and/or treat tumor recurrence and/or metastatic disease.

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Figure 1

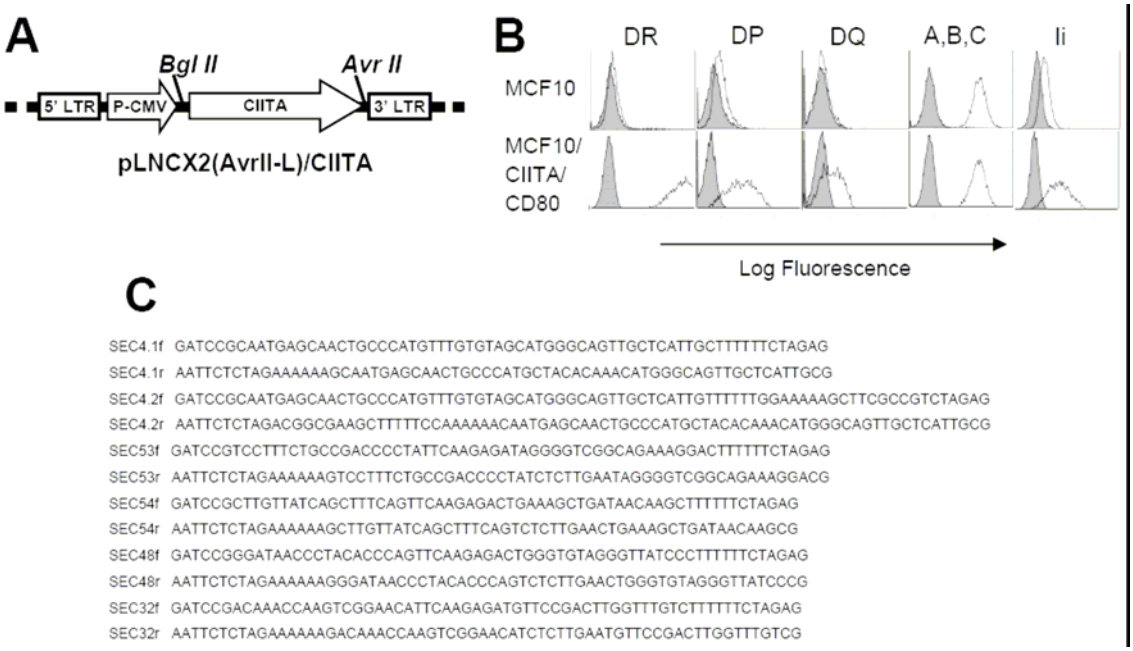


Figure 1. CIITA-transduced breast cancer cells express HLA-DR, HLA-DP, HLA-DQ, HLA-A, HLA-B, HLA-C, and invariant chain. A, CIITA retroviral vector. B, parental MCF10 and transduced MCF10/CIITA/CD80 cells were stained for HLA-DR (mAb L243), HLA-DQ (mAb CBL118P), HLA-DP (mAb CBL100F), HLA-A,B,C (mAb W6/32), or Ii (PIN1.1) and analyzed by flow cytometry. Unfilled peaks, specific antibody-stained cells; filled peaks, isotype control stained cells. C, forward (f) and reverse (r) oligonucleotides used to construct siRNA cassettes homologous to human Ii mRNA.

Figure 2

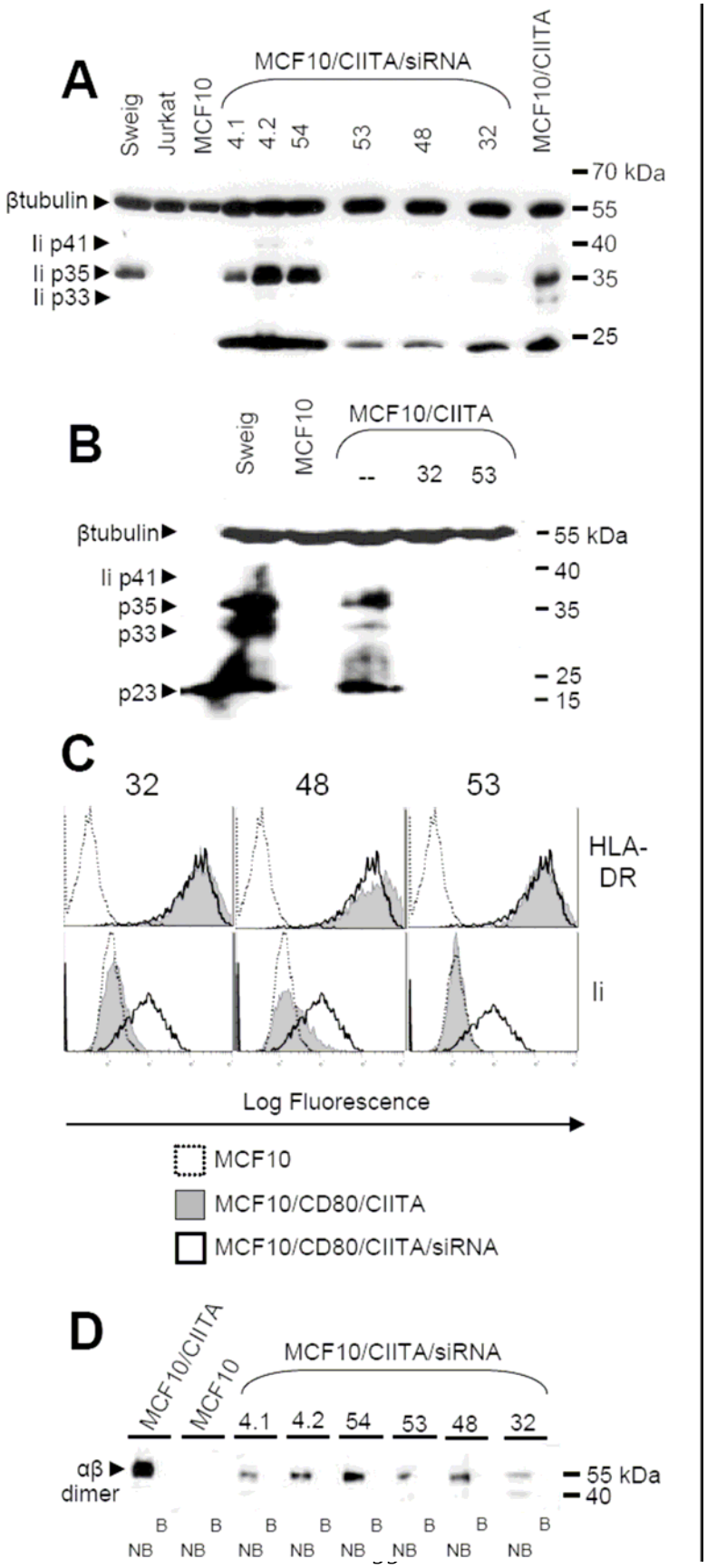

A
Sweig
Jurkat
MCF10
MCF10/CIITA/siRNA
4.1
4.2
54
53
48
32
MCF10/CIITA
70 kDa
βtubulin
55
li p41
40
li p35
35
li p33
25
B
Sweig
MCF10
MCF10/CIITA
--
32
53
βtubulin
55 kDa
li p41
40
p35
35
p33
p23
25
15
C
32
48
53
HLA-DR
li
Log Fluorescence
MCF10
MCF10/CD80/CIITA
MCF10/CD80/CIITA/siRNA
D
MCF10/CIITA
MCF10
MCF10/CIITA/siRNA
4.1
4.2
54
53
48
32
αβ dimer
55 kDa
40
B
NB

Figure 2. Ii siRNAs silence Ii expression in CIITA-transduced cells without altering HLA-DR expression. MCF10 cells were transduced with retroviruses containing the CIITA and siRNAs for Ii. A and B, Western blots of MCF10, transduced MCF10, and control Sweig and Jurkat cells probed for Ii with the PIN1.1 mAb, 3 days after transduction (A) or 3 weeks after transduction (B). C, parental and transduced MCF10 cells stained for HLA-DR (mAb L243) or Ii (mAb PIN1.1). D, Western blots of parental and transduced MCF10 cells probed for HLA-DR (mAb L243) after 3 weeks of drug selection. Samples were either boiled (B) or not boiled (NB) before loading on the gel. 4.1, 4.2, 32, 48, 53, 54 identify independent Ii siRNAs.

Figure 3

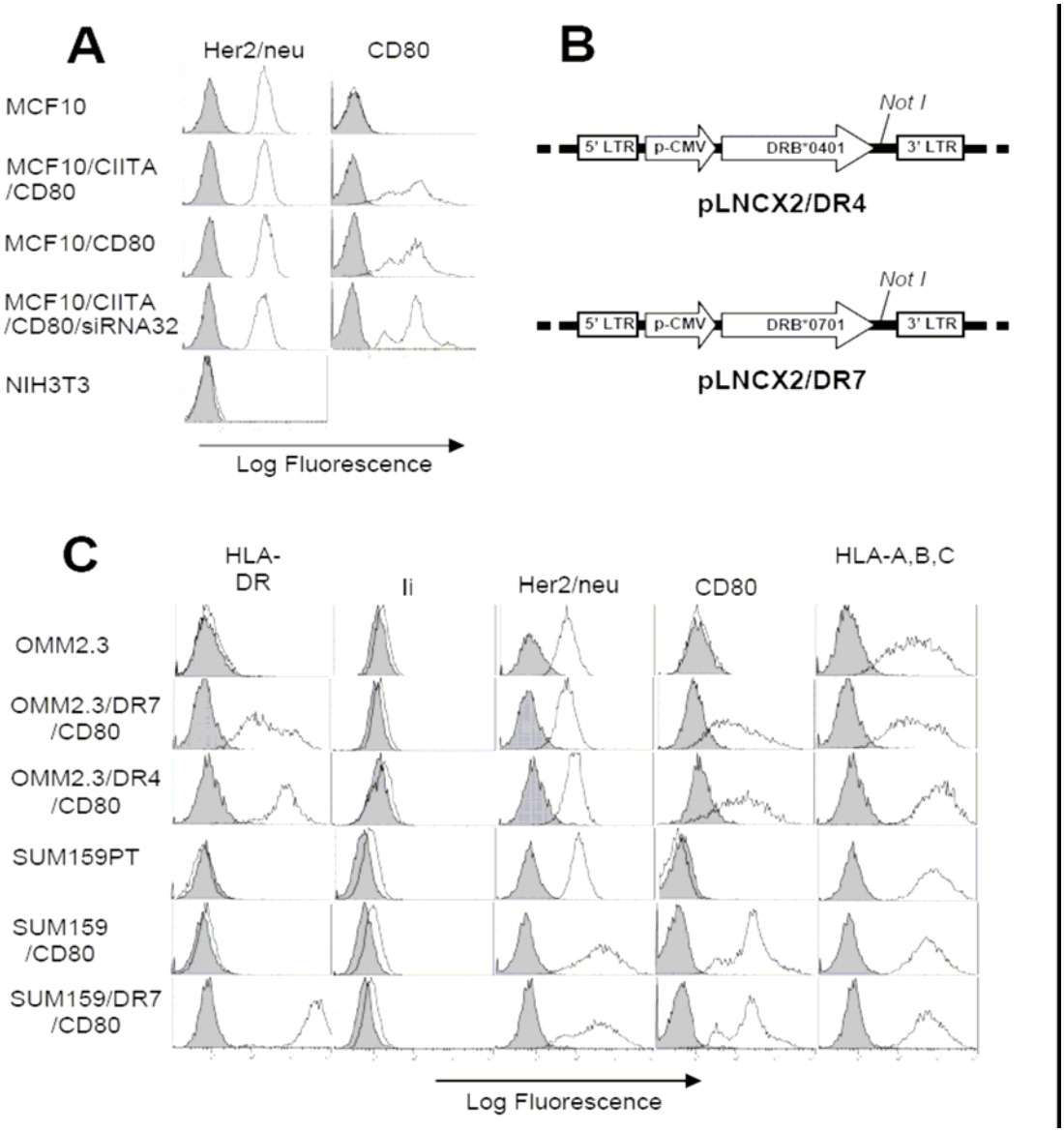
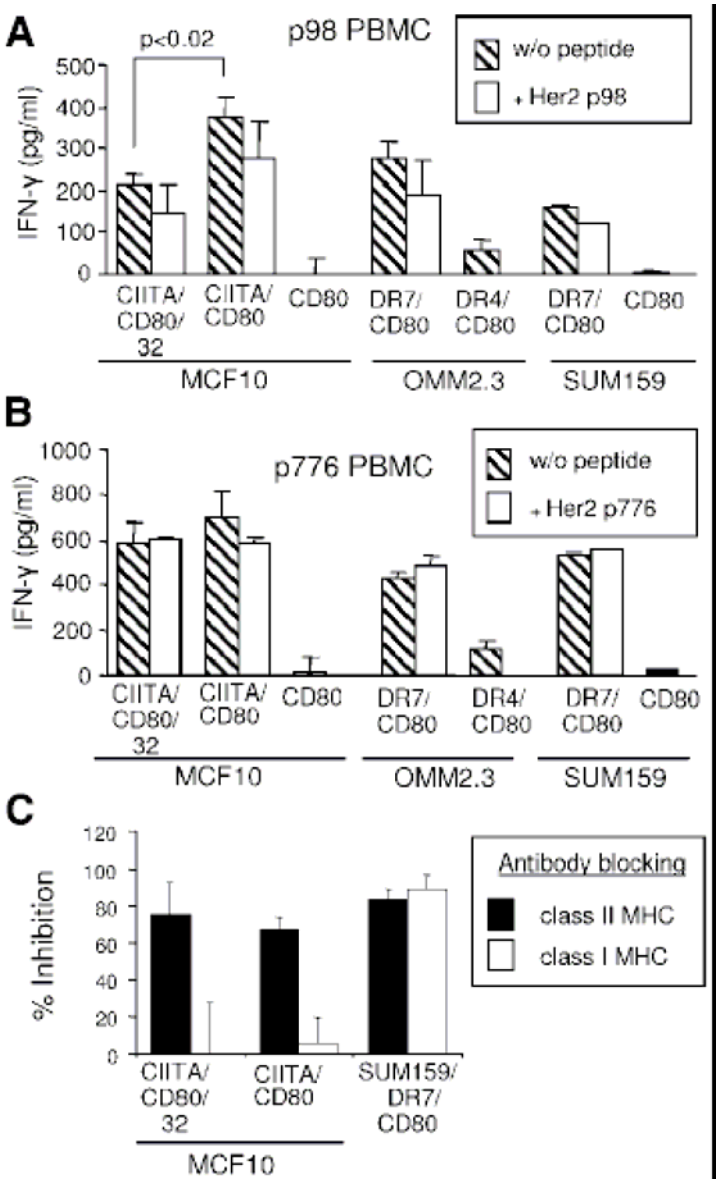


Figure 3. MCF10, OMM2.3, and SUM159PT cells overexpress HER-2/neu and transduced HLA genes and do not express Ii. A, parental and transduced MCF10 and control NIH3T3 cells labeled with antibodies to HER-2/neu or CD80 and analyzed by flow cytometry. B, HLA-DR4 and HLA-DR7 retroviral constructs. C, parental and transduced OMM2.3 and SUM159 cells stained with antibodies to HLA-DR (mAb L243), Ii (mAb PIN1.1), HER-2/neu, CD80, or HLA-A,B,C and analyzed by flow cytometry. Unfilled peaks, specific antibody-stained cells; filled peaks, isotype control stained cells.

Figure 4


A
p98 PBMC
p<0.02
w/o peptide
+ Her2 p98
IFN-γ (pg/ml)
500
400
300
200
100
0
CIITA/ CD80/ 32
CIITA/ CD80
CD80
DR7/ CD80
DR4/ CD80
DR7/ CD80
CD80
MCF10
OMM2.3
SUM159
B
p776 PBMC
w/o peptide
+ Her2 p776
IFN-γ (pg/ml)
1000
800
600
400
200
0
CIITA/ CD80/ 32
CIITA/ CD80
CD80
DR7/ CD80
DR4/ CD80
DR7/ CD80
CD80
MCF10
OMM2.3
SUM159
C
% Inhibition
120
100
80
60
40
20
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Antibody blocking
class II MHC
class I MHC
CIITA/ CD80/ 32
CIITA/ CD80
SUM159/ DR7/ CD80
MCF10

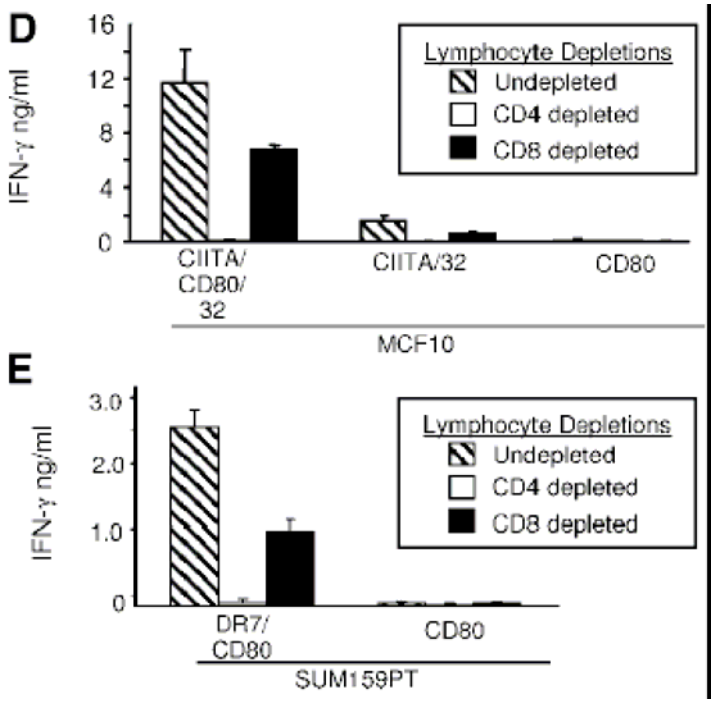

Figure 4. CIITA- and CD80-transduced MCF10 cells down-regulated for Ii by RNAi present endogenously synthesized HER-2/neu epitopes and activate CD4+ T cells. HLA-DR7-restricted peptide 98–specific T cells (A) or peptide 776–specific T cells (B) were cocultured with MCF10, OMM2.3, or SUM159PT parental cells or transductants, and T-cell activation was quantified by measuring IFN-g release. Exogenous peptide was added to some wells. C, p98- and p776-primed T cells were cocultured with transduced MCF10 or SUM159PT cells in the presence of antibodies to HLA-DR (mAb L243) or HLA-A,B,C (mAb W6/32). HLA-DR7-restricted, p776-primed PBMCs were depleted for CD4+ or CD8+ T cells before incubation with MCF10 (D) or SUM159PT (E) transductants as in (A) and (B).

Figure 5

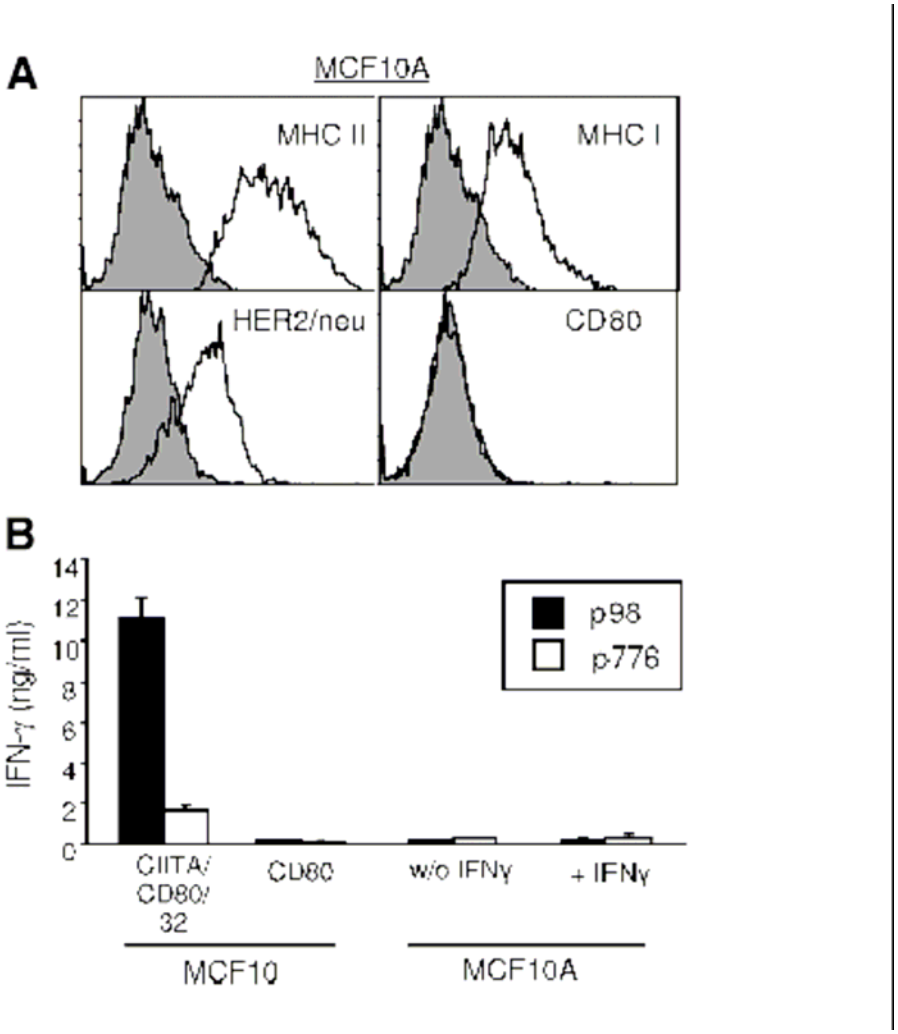

Figure 5. Nonmalignant breast cells do not activate T cells. A, untreated MCF10A cells were stained for CD80 or HER-2/neu, and IFN-g-treated (1,000 units for 48 hours) MCF10A cells were stained for HLA-DR or HLA-A,B,C and analyzed by flow cytometry. Unfilled peaks, specific antibody-stained cells; filled peaks, isotype control stained cells. B, PBMCs were pulsed with p776 or p98 and incubated with MCF10, MCF10A, or IFNg-treated MCF10A cells, and the supernatants were analyzed for IFN-g as per Fig. 4.

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Chapter 4

The Absence of Invariant Chain in MHC II Cancer Vaccines Enhances the Activation of Tumor-Reactive Type 1 $CD4^+$ T Lymphocytes

In review: James A. Thompson, Minu K. Srivastava, Jacobus J. Bosch, Bruce R. Ksander, Suzanne Ostrand-Rosenberg. The Absence of Invariant Chain in MHC II Cancer Vaccines Enhances the Activation of Tumor-Reactive Type 1 CD4+ T Lymphocytes. Cancer Res.

The Absence of Invariant Chain in MHC II Cancer Vaccines Enhances the Activation of Tumor-Reactive Type 1 $CD4^+$ T Lymphocytes

by

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Running title: $MHCII^+Ii^-$ cancer vaccines prime and boost $CD4^+$ T cells

Key Words: cancer vaccines, major histocompatibility complex class II, antigen processing and presentation

Abstract

Activation of tumor-reactive T lymphocytes is a promising approach for the prevention and treatment of patients with metastatic cancers. Strategies that activate $CD8^+$ T cells are particularly promising because of the cytotoxicity and specificity of $CD8^+$ T cells for tumor cells. Optimal $CD8^+$ T cell activity requires the co-activation of $CD4^+$ T cells, which are critical for immune memory and protection against latent metastatic disease. Therefore, we are developing "MHCII" vaccines that activate tumor-reactive $CD4^+$ T cells. MHCII vaccines are MHC class I^+ tumor cells that are transduced with costimulatory molecules and MHCII alleles syngeneic to the prospective recipient. Because the vaccine cells do not express the MHCII-associated invariant chain (Ii), we hypothesized that they will present *endogenously* synthesized tumor peptides that are not presented by professional Ii^+ antigen presenting cells (APC) and will therefore overcome tolerance to activate $CD4^+$ T cells. We now report that MHC II vaccines prepared from human MCF10 mammary carcinoma cells are more efficient than Ii^+ APC for priming and boosting Type 1 $CD4^+$ T cells. MHCII vaccines consistently induce greater expansion of $CD4^+$ T cells which secrete more IFNγ and they activate an overlapping, but distinct repertoire of $CD4^+$ T cells as measured by T cell receptor Vβ usage, compared to Ii^+ APC. Therefore, the absence of Ii facilitates a robust $CD4^+$ T cell response that includes the presentation of peptides that are presented by traditional APC, as well as peptides that are uniquely presented by the Ii^- vaccine cells.

Introduction

Successful cancer vaccines must induce potent tumor-specific immunity and long term immunological memory to protect against recurrent tumors and metastatic disease. Many vaccines are aimed at boosting cell-mediated immunity, which is considered particularly promising due to its ability to destroy tumor cells. Type 1 $CD4^+$ T cells, which secrete IFNγ, have long been recognized as critical components for the activation of $CD8^+$ T cells, either through their classical role as "helper" T cells that provide cytokine support for $CD8^+$ T cells [6, 22], or by their induction of CD40 expression on dendritic cells (DC) ("licensing"), which in turn activate $CD8^+$ T cells [5, 36, 40]. $CD4^+$ T cells are also essential for generating $CD8^+$ T memory cells, for preventing $CD8^+$ T cells from being tolerized [3, 19-21, 41, 44, 45], and for recruiting cells of the innate immune system, such as macrophages. IFNγ production at the tumor site also up-regulates tumor-expressed MHC molecules to improve CTL recognition, and blocks neo-vascularization to prevent tumor proliferation [18, 34, 35]. Because of the central role of $CD4^+$ T cells in facilitating anti-tumor immunity, we are developing vaccine strategies that specifically activate $CD4^+$ T cells.

Activation of $CD4^+$ T cells requires two signals: an antigen-specific signal delivered by a major histocompatibility complex class II (MHC II) molecule presenting a specific peptide, and a costimulatory signal. Expression of MHC II and costimulatory molecules is usually limited to professional antigen presenting cells (APC), such as DC. Therefore, activation of tumor-reactive $CD4^+$ T cells requires that tumor antigens are endocytosed by DC and processed and presented to the appropriate $CD4^+$ T cells [37]. Presentation of exogenously acquired antigen by DC is facilitated by the expression of the MHC II accessory molecule invariant chain (Ii). As newly

synthesized MHC II molecules enter the endoplasmic reticulum (ER), their peptide-binding groove is occupied by Ii. From the ER, MHC II/Ii complexes traffic to endosomal compartments where Ii is degraded, and peptides derived from endocytosed, exogenously synthesized molecules are loaded with the help of HLA-DM [10]. As a result, $CD4^+$ T cells that are activated by professional APC react with antigens obtained from third party (e.g. tumor) cells, but the actual peptides for which the $CD4^+$ T cells are specific, are generated by DC.

Tumors synthesize antigens that are immunogenic and under some circumstances can activate an immune response that mediates tumor rejection [16, 38]. However, many tumor-bearing individuals are tolerized to the tumor-derived peptides presented by their DC, and do not produce tumor-reactive $CD4^+$ T cells [42, 43].

We are developing cell-based "MHC II vaccines" that are designed to overcome an individual's tolerance to their tumor peptides. The MHC II vaccines are tumor cells that are genetically modified to express MHC II and costimulatory molecules. In professional APC which are Ii^+, peptides derived from *exogenously* synthesized molecules are processed in endosomes where they bind to MHC II molecules. Because the vaccine cells do not express Ii, we have hypothesized that MHC II molecules bind peptides in the ER that are derived from *endogenously* synthesized proteins [1, 32, 33]. Since ER-resident peptides are likely to differ from endosomally generated peptides [9], the vaccines may present novel peptides that are not presented by professional Ii positive APC. Therefore, the vaccine cells should activate a different population of $CD4^+$ T cells than that activated by Ii positive APC, thereby overcoming tolerance and stimulating tumor-specific immunity.

In previous reports MHC II vaccines were shown to have significant therapeutic efficacy in mouse models against large, established primary [2] and spontaneously metastatic [30, 31]

tumors. We have recently adapted the MHC II vaccine approach for human tumors and have developed several vaccines for human mammary carcinoma and ocular melanoma [13]. Similar to the mouse vaccines, the human MHC II vaccines efficiently present endogenously synthesized tumor peptides, and activate tumor-reactive $CD4^+$ T cells from healthy donors [47] and from patients with primary or metastatic cancer [8]. In this report we demonstrate that human Ii negative MHC II vaccine cells are significantly better activators of tumor-reactive Type 1 $CD4^+$ T cells than are Ii positive APC, and that the Ii negative vaccine cells activate an overlapping, but distinct, repertoire of $CD4^+$ T cells than is activated by Ii positive APC. These findings support our hypothesis that Ii negative MHC II vaccine cells present some tumor peptides that are not presented by professional Ii positive APC and therefore may be effective therapeutic and/or prophylactic agents for cancer patients.

Materials and Methods

Cells. The human tumor cell lines Sweig and Jurkat, peripheral blood mononuclear cells (PBMC) from healthy human donors [13], MCF10CA1 human mammary carcinoma (hereafter called MCF10), and MCF10 transductants [47] were handled as described. MCF10/CIITA/CD80/Ii siRNA cells are MCF10/CIITA/CD80 cells that were rendered Ii negative by transduction with Ii siRNA 32. MHC II and Ii siRNA transductants were previously shown to express stable MHC II αβ heterodimers and not express Ii as assayed by western blotting and flow cytometry [47]. PBMC were obtained by venipuncture from healthy human donors and buffy coats were stored at -80^{o}C until used. Thawed PBMC were >90% viable. All

cell lines and procedures were approved by the institutional review boards of the participating institutions.

Retroviral constructs, transductions and drug selection. The human major histocompatibility complex class II associated invariant chain (Ii) cDNA (Genbank accession no. NID X00497), containing both start codons for p33 and p35 isoforms of Ii, was cloned from sp64/p33 (kindly provided by Eric Long, NIH) [23], into the pLPCX vector (Clontech) containing a puromycin resistance gene. To construct the pLPCX/Ii vector containing the Ii gene, Ii was excised from sp64/p33 with *SalI* and *EcoRI* (New England Biolabs; Beverly, MA) and inserted into the pLPCX vector digested with *XhoI* (New England Biolabs) and *EcoRI* (*XhoI* having compatible cohesive ends with *SalI*). Proper insertion of the Ii gene was confirmed by sequence analysis. Sequencing reactions were carried out using the 'ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1' (Perkin Elmer Applied Biosystems Division, Foster City, CA) according to the manufacturer's protocol using a PTC-200 Thermal Cycler (MJ Research, BioRad, Hercules, CA) and an ABI PRISM 3700 DNA Analyzer (Applied Biosystem). Retroviruses were prepared, and tumor cells were transduced with the retroviruses as described [13]. Transduced cells were selected in 0.3 g/ml puromycin for 2-3 weeks after transduction, and thereafter maintained in MCF10 medium without puromycin. Transduced cells were periodically monitored by flow cytometry for expression of the transduced genes, which were stably expressed for >12 months.

Antibodies, immunofluorescence, western blots. HLA-DR-FITC, CD80-PE, FITC-isotype and PE-isotype controls were from BD Biosciences. CD4-FITC and CD8-FITC were from Miltenyi Biotec Inc. (Auburn, CA); HLA-DM-PE was from BDBiosciences (San Diego, CA). Culture supernatants of hybridomas W6/32 (pan HLA-A, B, C), L243 (pan anti-HLA-DR), and PIN1.1 (anti-Ii) were prepared, and tumor cells and PBMC were stained for cell surface markers, or fixed and stained for Ii as described [13]. T cell receptor (TCR) V repertoire of $CD4^+$ T cells was determined by three color flow cytometry analysis using the IO Test Beta Mark TCR V Repertoire kit (Beckman Coulter, San Diego, CA), which consists of monoclonal antibodies (mAbs) to 24 distinct TCR V families (about 70% coverage of normal human TCR V repertoire). Each test includes three directly coupled TCR V mAbs, conjugated to PE, FITC or both PE and FITC. PBMC were stained and analyzed for TCR V usage according to the manufacturer's instructions, with the following modification: prior to staining with V mAbs, 1×10^5 PBMC were stained with 1μg of biotin conjugated anti-human CD4 (eBiosciense, SanDiego, CA), then washed and stained with streptavidin-PerCP (BD Biosciences, San Jose, CA). Viable $CD4^+$ T cells were gated according to CD4 staining and side scatter, and this population was analyzed for V TCR usage. Western Blots were done as described eld MACROBUTTON endnote+.cit [47].

T-cell priming and T cell depletions. Transduced MCF10, (MCF10/DR7/CD80, MCF10/DR7/CD80/Ii, MCF10/CIITA/CD80, and MCF10/CIITA/CD80/Ii siRNA) cells were plated on day 0 in MCF10 medium at $2\text{-}4\times10^5$ cells/4mls in 6cm dishes and the cells allowed to attach before irradiating with 25 Gy. The next morning MCF10 medium was removed and $1\text{-}2\times10^7$ PBMC in 4 ml of T cell medium (Isocoves Modified Dulbeccos Medium, 1%gentamicin,

1% penicillin, 2mM Glutamax, 0.01M HEPES, 1mM sodium pyruvate, (Invitrogen, Carlsbad, CA), 0.01M -mercaptoethanol (VWR West Chester, PA), 5% human AB serum (Genini Bio-Products Woodland, CA)) were added to each 6 cm dish, and the cells were incubated at 37^{o} C for 3-5 days. At the end of this incubation period, non-adherent cells (PBMC) were harvested, washed twice, and resuspended at 1×10^{6} PBMC/2ml T cell medium supplemented with human recombinant IL-15 (40 ng/2ml) (Peprotech, Rocky Hill, NJ). PBMC were then plated in 24 well plates at 1×10^{6} cells/2ml/well. After a 7 day culture period, the non-adherent cells ("primed PBMC") were removed, washed twice, and then rested in T-cell medium for 1-3 days before being used in antigen presentation assays. In some experiments, PBMC were depleted for $CD4^{+}$ or $CD8^{+}$ T cells before priming, using LD columns and CD4 or CD8 microbeads according to the manufacturers' directions (Miltenyi, Auburn, CA).

Antigen presentation assays. MHC II vaccine cells were plated at 2.5×10^{4} cells/200 l MCF10 medium/well in 96 well plates and allowed to adhere for 2-4 hours. MCF10 medium was then removed and primed PBMC in T cell medium were added at 5×10^{4} PBMC/200 l/well. After 48 hours of incubation, the supernatants were analyzed for IFNγ by ELISA [13], and the non-adherent cells were analyzed for TcR V repertoire by flow cytometry. Multiplex cytokine analyses were done in the Cytokine Core Facility at the University of Maryland, Baltimore using the Luminex 100 System (Luminex, Austin, TX) according to the manufacturer's directions.

HLA Haplotyping. PBMC from healthy donors and MCF10 cell lines were HLA typed using SSP-ABDR DNA typing trays (OneLambda, Canoga Park, CA) according to the manufacturer's recommendations. MCF10 are A33, B55, DR7. PBMC 100704 are A11, A29;

B44, B51; DR4, DR7, DR53. PBMC 111504 are: A1, A68; B35, B57; DR1, DR7, DR53; PBMC 123104 are: A33, A36; B-, B44; DR1, DR7, DR53.

Statistical analysis and TCR Vβ usage calculations. Means, SD, and statistical significance as measured by Student's t test were calculated using Excel v2002. Percent increase in TCR Vβ usage = 100% × [(% cells with specific TCR Vβ after activation with Ii^- or Ii^+ APC)/(% cells with specific TCR Vβ before activation)].

Results

MCF10 human mammary carcinoma cells transduced with an Ii (p33) expression vector express Ii. To study the effects of Ii on the repertoire of peptides presented by MHC II-transduced human breast cancer cells, we have constructed an expression vector that contains p33, which is the dominant isoform of Ii [23] (**Fig.1*A***). MCF10 cells previously transduced with retroviruses encoding MHC II and CD80 molecules (MCF10/DR7/CD80) were further transduced with Ii retroviruses. The resulting cells (MCF10/DR7/CD80/Ii) were stained with mAbs for cell surface expression of HLA-DR, HLA-DM, and CD80 (live cells), or for internal Ii expression (fixed cells), and analyzed by flow cytometry (**Fig. 1*B***). MCF10/DR7/CD80/Ii cells express Ii protein, and their levels of HLA-DR and CD80 are similar to the levels expressed by Ii^- MCF10/DR7/CD80 cells. Neither population expresses HLA-DM. Expression of the Ii p33 isoform by MCF10/DR7/CD80/Ii cells was further confirmed by western blotting using the human B cell lymphoma cell line Sweig as a positive control for p33 (**Fig. 1*C***). Therefore, Ii is

expressed in MCF10/DR7/CD80/Ii breast cancer cells, and its expression does not alter expression of HLA-DR7, HLA-DM, or CD80.

Ii^- transductants prime $CD4^+$ T cells more efficiently than Ii^+ transductants.

Previous studies have established that Ii^- MCF10/DR7/CD80 transductants bind MHC II-restricted peptides and activate $CD4^+$ T cells that were primed by peptide pulsing; however, these studies did not determine if the Ii^- vaccines prime $CD4^+$ T cells [47]. To test whether Ii^- vaccine cells efficiently prime $CD4^+$ T cells, HLA-$DR7^+$ PBMC from healthy human donors were depleted for $CD8^+$ T cells and co-cultured (primed) with irradiated MCF10/DR7/CD80 vaccine cells. Following three days of culture, non-adherent cells (lymphocytes) were removed, expanded in IL-15 for six days, re-cultured (boosted) with MCF10/CD80 or MCF10/DR7/CD80 cells, and the supernatants analyzed for IFNγ (**Fig. 2*A***). Boosting with MCF10/DR7/CD80, but not with the MCF10/CD80, cells induces IFNγ release, indicating that the transduced MHC II allele (DR7) is essential for priming of $CD4^+$ T cells and that allogeneic MHC I differences between the vaccine cells and the responding PBMC do not result in IFNγ release. Therefore, the vaccine cells prime naçve $CD4^+$ T cells and the priming is MHC II-dependent.

To determine if Ii^+ cells are as effective as the Ii^- vaccines in priming, $DR7^+$ PBMC from healthy donor 100704 were primed with Ii^- (MCF10/DR7/ CD80) or with Ii^+ (MCF10/DR7/CD80/Ii) cells, expanded in IL-15, rested, and boosted with the transductants used for priming. PBMC were either not depleted, or depleted for $CD4^+$ or $CD8^+$ T cells prior to priming (**Fig. 2*B***). Ii^- vaccine cells induced more than twice as much IFNγ production as Ii^+ cells and the response is virtually eliminated by depletion of $CD4^+$ T cells prior to priming, indicating that $CD4_+$ T cells are the only cells activated to produce IFN(. Priming and boosting

with Ii^- cells also gave 1.5-2 fold more $CD4^+$ T cells compared to priming and boosting with Ii^+ cells (data not shown).

To ascertain the type of response induced by the Ii^- vaccine cells, the supernatants from the boosted cultures of **Fig. 2*B*** were assayed by multiplex analysis for IL-2, IL-4, IL-10, IL-15, and IFNγ (**fig. 2*C***). MCF10/DR7/CD80 vaccine cells induce high levels of the type 1 cytokine IFNγ, intermediate levels of IL-2, and very low levels of the type 2 cytokines IL-4 and IL-10. Therefore, MCF10/DR7/CD80 vaccine cells are significantly more efficient than Ii^+ cells for priming type 1 tumor-reactive $CD4^+$ T lymphocytes.

HLA-DM expression does not affect $CD4^+$ T cell activation by MHC II vaccine cells. In professional APC such as DC, HLA-DM facilitates the binding of peptides derived from exogenously synthesized molecules by displacing the class II-associated Ii peptide (CLIP) from the peptide binding groove of MHC II molecules [10]. Since MCF10/DR7/CD80/Ii vaccine cells contain Ii, but do not contain DM (**Fig. 1B**), their reduced antigen presentation activity may be due to an inability to replace CLIP with other peptides. If MCF10/DR7/CD80/Ii cells have this defect and are unable to replace CLIP, then their cell surface HLA-DR molecules will have bound CLIP. To assess this possibility, live MCF10/DR7/CD80/Ii cells were stained with the CerCLIP mAb which detects MHC II/CLIP complexes (**Fig. 3A**). MCF10/DR7/CD80/Ii cells express approximately the same amount of HLA-DR/CLIP complexes at the cell surface as the B cell lymphoma Sweig, which constitutively expresses HLA-DR, Ii, and DM. As expected, MCF10/DR7/CD80 cells do not express HLA-DR/CLIP complexes at the cell surface since they do not contain Ii. Therefore, MCF10/DR7/CD80/Ii cells do not express significantly higher levels of CLIP than Sweig cells, making it unlikely that their reduced antigen presentation

activity is due to excessive levels of CLIP interfering with MHC II-restricted peptide presentation.

Because professional APC such as DC express DM, we have prepared MHC II vaccines using the MHC class II transactivator (CIITA), which coordinately up-regulates MHC II, Ii, and DM [15], and an siRNA to down-regulate Ii (MCF10/CIITA/CD80/Ii siRNA cells) [47]. As measured by flow cytometry, these vaccine cells express high levels of HLA-DR, very low levels of HLA-DR/CLIP complexes at the cell surface (**Fig. 3A**), and levels of HLA-DM similar to Sweig cells (**Fig. 3B**). Since PBMC that are syngeneic to the MCF10 vaccines are not available, and CIITA up-regulates all HLA-DR, -DP, and DQ alleles, the CIITA transductants cannot be used as priming agents because they will induce allogeneic responses. However, allogeneic T cell activation should not occur during the short duration of the boosting response, so the CIITA transductants can be used as boosting agents. To determine if DM expression impacts the ability of Ii^- vaccine cells to boost $CD4^+$ T cells, $DR7^+$ PBMC from healthy donor 111504 were depleted of $CD8^+$ T cells, primed with Ii^- MCF10/DR7/CD80 cells, and boosted with Ii^-DM^- (MCF10/DR7/CD80), Ii^-DM^+ (MCF10/CIITA/CD80/Ii siRNA), or Ii^+DM^+ (MCF10/CIITA/CD80) cells (**Fig. 4*A*).** Boosting with Ii^-DM^- or Ii^-DM^+ cells induces a strong response, while boosting with Ii^+DM^+ cells gives a much poorer response. As expected, allogeneic MHC I differences between the boosting cells and responding PBMC do not activate $CD4^+$ T cells because MCF10/CIITA/CD80 cells do not cause significant IFNγ release. Therefore, DM expression does not impact the boosting of $CD4^+$ T cells that are primed with Ii^- vaccine cells.

Although DM expression is irrelevant for the boosting of $CD4^+$ T cells that are primed with Ii^- cells, it may affect the boosting of $CD4^+$ T cells that are primed with Ii^+ cells. To test

this possibility, $DR7^+$ PBMC from healthy donor 123104 were depleted for $CD8^+$ T cells and primed with Ii^- MCF10/DR7/CD80 or Ii^+ MCF10/DR7/CD80/Ii cells and boosted with Ii^-DM^- (MCF10/DR7/CD80), Ii^+DM^- (MCF10/DR7/CD80/Ii), Ii^-DM^+ (MCF10/CIITA/CD80/Ii siRNA), or Ii^+DM^+ (MCF10/CIITA/CD80) cells (**Fig. 4*B*).** Ii^- cells are significantly more efficient than Ii^+ cells for boosting $CD4^+$ T cells, regardless of DM expression. Therefore, Ii^- MHC II vaccine cells are more effective activators of $CD4^+$ T cells than Ii^+ cells, and the decreased efficiency of the Ii^+ cells is unlinked to HLA-DM expression.

Ii^- and Ii^+ vaccine cells activate overlapping, but distinct, repertoires of $CD4^+$ T cells. If Ii^- vaccine cells present novel tumor antigen epitopes that are not presented by Ii^+ cells, then Ii^- and Ii^+ cells should activate different repertoires of $CD4^+$ T cells. This hypothesis was tested by assessing the TCR Vβ repertoires of the $CD4^+$ T cells activated by Ii^+ vs. Ii^- cells. PBMC from $DR7^+$ healthy human donors were primed and boosted with Ii^- (MCF10/DR7/CD80) or Ii^+ (MCF10/DR7/CD80/Ii) cells, and the non-adherent cells were harvested and triple-stained for CD4 and 24 different TcR V s. The $CD4^+$ T cells were gated **(Fig. 5*A*)** and analyzed for Vβ expression. **Fig. 5*B*** shows the TCR Vβ family usage by $CD4^+$ T cells from healthy donors 123104 and 100704 before and after activation with either the Ii^- or Ii^+ cells. **Table 1** shows the number of TCR V∃ families that are increased at least 20% in response to Ii^- or Ii^+ cells in these two donors plus a third healthy donor (111504). Some TCR V∃ families are equally activated by both Ii^- and Ii^+ cells, while other families are activated exclusively by Ii^- or Ii^+ APC. Therefore, Ii^- and Ii^+ cells activate some of the same TCR V∃ families, but they also activate distinct families, demonstrating that Ii^- vaccine cells activate $CD4^+$ T cells that are not activated by standard APC that are Ii^+.

Discussion

The human MHC II vaccines described in this report are generated by transducing Ii^- MHC I^+ mammary carcinoma cells with CD80 and MHC II alleles syngeneic to the prospective recipient. Previous studies demonstrated that such vaccines induce tumor-specific immunity that results in rejection of established primary and metastatic disease in mouse models [2, 12, 29, 31]. This report extends the mouse studies to humans and demonstrates that the absence of Ii in the vaccine cells is a critical element for vaccine efficacy. We find that MHC II vaccines prime and boost type 1 $CD4^+$ T cells in vitro to release high levels of IFNγ. MHC II vaccines consistently induced greater expansion of $CD4^+$ T cells that secreted more IFNγ and expressed a different repertoire of T cell receptor gene families than $CD4^+$ T cells primed and/or boosted by the same cells expressing Ii. Collectively, these observations are consistent with the hypothesis that the absence of Ii facilitates a more robust $CD4^+$ T cell response that includes the presentation of tumor peptides that are presented by Ii^+ APC, as well as distinct peptides that are uniquely presented by Ii^- APC.

There are several possible explanations why the absence of Ii promotes greater expansion of T cells and increased IFNγ secretion by activated $CD4^+$ T cells. In contrast to professional APC, the peptide binding groove of newly synthesized MHC II molecules of MHC II vaccines is not occupied by Ii or CLIP, and may bind peptides derived from endogenously synthesized molecules either in the ER or as they traffic from the ER to endosomes. If the ER contains peptides that are not present in endosomes, then the genetically modified tumor cells may be presenting peptides that are not presented by professional APC. Such antigen presentation would

result in the activation of $CD4^+$ T cells that are not typically activated by professional Ii^+ APC. Other studies using different experimental approaches support this possibility. For example, Ii^- MHC II vaccine cells activate $CD4^+$ T cells to peptides derived from diverse subcellular compartments, whereas Ii^+ APC do not [33], consistent with the concept that Ii^- MHC II vaccine cells present a different repertoire of tumor peptides than the repertoire presented by Ii^+ APC. Studies with Ii knockout mice similarly show that the absence of Ii facilitates the presentation of novel endogenous epitopes, while most endogenous antigens that are presented in the presence of Ii are also presented in the absence of Ii [7]. These findings are also corroborated by biochemical studies in which mass spectroscopy analysis of MHC II-bound peptides demonstrated that Ii^- APC display peptides that are not presented by Ii^+ APC [28].

The presentation of different and/or more immunogenic epitopes by Ii^- MHC II vaccines may also be due to the presentation of "cryptic" peptides, a term coined by investigators studying autoimmunity. Cryptic peptides are peptides that are not presented in the thymus during central tolerance induction, but are present in the periphery and which bind with high affinity to MHC molecules. Such peptides have been identified and shown to induce T cell responses to self antigens [4, 17, 24, 39]. If the absence of Ii in MHC II vaccine cells results in altered antigen processing and presentation, as we have hypothesized, then the vaccine cells may present cryptic tumor peptides which potentially induce a more robust $CD4^+$ T cell response.

The MCF10/DR7/CD80 and MCF10/CIITA/CD80/siRNA vaccines both activate tumor-reactive $CD4^+$ T cells in the absence of Ii and have the potential to be clinically useful. We have proposed using the vaccines in non-autologous patients who are HLA-DR-matched to the vaccine cells, to avoid customized vaccine preparation for individual patients [8]. Each vaccine has its pros and cons. Advantages of the multiple allele CIITA-based vaccines include a) their

presentation of tumor peptides on multiple MHC II alleles; b) their ease of production via a universal construct encoding the CIITA, Ii siRNA, and CD80; c) the potential to use tumor cells that constitutively express MHC II; and d) increased expression of MHC class I molecules due to up-regulation of MHC I by the CIITA. A drawback of the CIITA vaccines is their potential to activate allo-MHC II responses, which may overwhelm the tumor-specific MHC II-restricted response in non-fully MHC II-matched recipients. A major advantage of the single allele vaccines is the ease of HLA-DR-matching of the vaccine to an individual patient. A potential disadvantage of the single allele vaccines is that an IFN(-inducible tumor cell line could be upregulated for Ii if the vaccine enters or induces an inflammatory environoment. However, this disadvantage can be overcome if non-IFN(-inducible cells are used or if the cells are co-transduced with Ii siRNA. Both the single allele and multiple allele vaccines could be stored as frozen cells, thereby minimizing reagent preparation time for individual patients.

Several clinical observations support our hypothesis that tumor cell co-expression of Ii with MHC II blocks tumor cell immunogenicity and favors tumor progression by impeding T cell activation to endogenously synthesized tumor antigens. Chamuleau and colleagues have observed that acute myelogenous leukemia patients whose myeloid leukemic blasts are HLA-$DR^{+}CLIP^{-\text{ or low}}$ have a significantly better clinical prognosis than patients whose blasts are $DR^{+}CLIP^{high}$ [11]. Since the failure to remove CLIP from MHC II molecules reduces endogenous antigen presentation [25], this observation is consistent with the concept that HLA-DR expression in the absence of Ii favors the activation of tumor-reactive $CD4^{+}$ T cells. Similarly, the co-expression of Ii by HLA-DR^{+} hepatocellular carcinoma cells is associated with a very poor prognosis [46]. Likewise, tumor cell expression of an isoform of Ii that blocks endogenous antigen presentation is associated with poor prognosis in chronic lymphocytic

leukemia patients [48]. If the vaccine cells are the actual APC in vivo, then they may be particularly useful for treating cancer patients whose DC are dysfunctional due to tumor burden [26, 27]. If the vaccine cells are not the relevant APC in vivo, and $CD4^+$ T cells are activated via cross-dressing [14] or by conventional antigen presentation via DC, then the vaccines are useful reagents for producing novel peptide-MHC complexes. Regardless of the precise mechanism by which the MHC II vaccines activate tumor-reactive $CD4^+$ T cells, they efficiently prime and boost $CD4^+$ T cells, and the absence of Ii is critical for their activity.

Acknowledgments

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Table 1. Ii^- and Ii^+ breast cancer cells activate over-lapping and distinct repertoires of $CD4^+$ T cells.

PBMC donor	Number of TCR Vβ families expanded after activation[a]		
	Ii^+ APC	Ii^- APC	Ii^+ and Ii^- APC[b]
123104	1	2	6
100704	1	3	10
111504	1	6	5

[a] Expansion of a given TCR Vβ family is defined as a statistically significant increase ($p< 0.5$) of at least 20% in the percent of $CD4^+$ T cells after priming and boosting compared to unprimed $CD4^+$ T cells.

[b] Number of common TCR Vβ families expanded by both Ii^- and Ii^+ APC

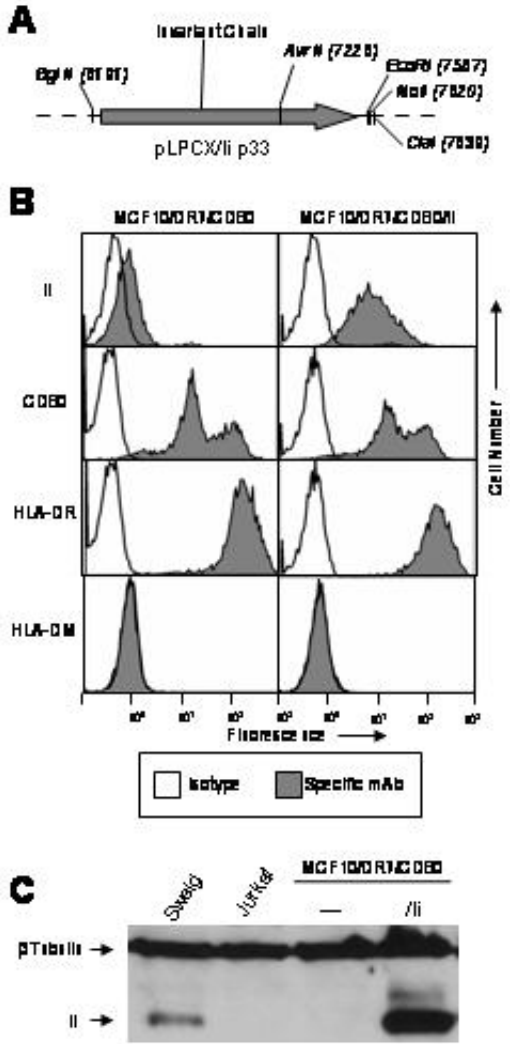

Fig. 1. MHC II breast cancer vaccines prepared from MCF10 cells express HLA-DR and CD80 and do not express invariant chain unless transduced with a p33 Ii construct. ***A***, Invariant chain retroviral vector. ***B***, MCF10/DR7/CD80 and MCF10/DR7/CD80/Ii transductants stained for Ii (mAb Pin1.1), CD80 (mAb CD80), HLA-DR (mAb L243), or HLA-DM (mAb DM), and analyzed by flow cytometry. ***C***, Western blot of MCF10/DR7/CD80 and MCF10/DR7/CD80/Ii cells and control Sweig and Jurkat cells probed for Ii with the PIN1.1 mAb. Data of panels B and C are representative of 3 independent experiments.

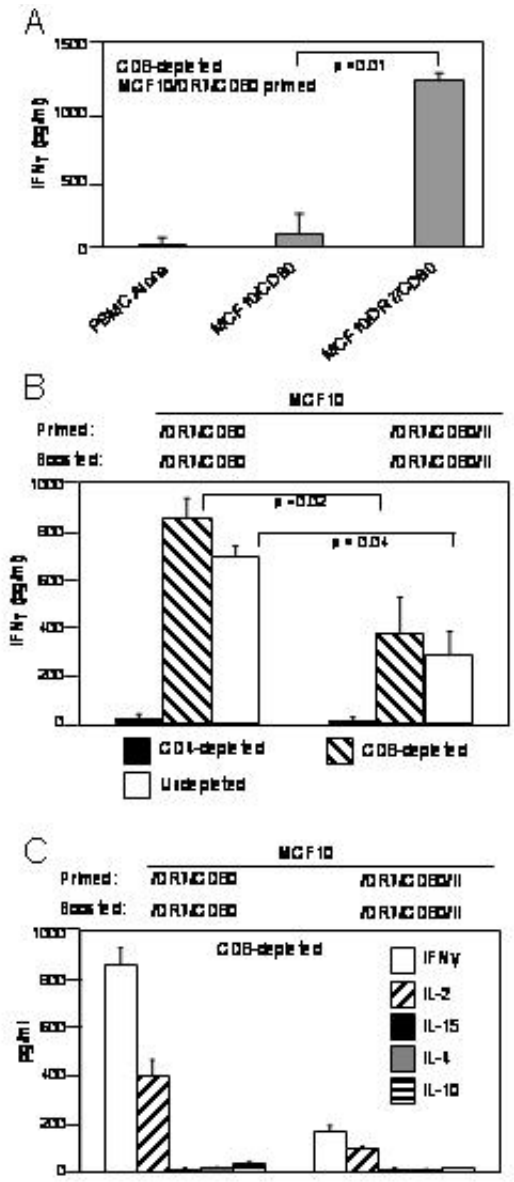

Fig. 2. MCF10/DR7/CD80 vaccine cells prime and boost healthy donor PBMC and are better activators of $CD4^+$ T cells than MCF10/DR7/CD80/Ii cells. ***A***, PBMC from healthy donor 100704 were depleted for $CD8^+$ T cells, primed with MCF10/DR7/CD80 vaccine cells, boosted with MHC II negative control (MCF10/CD80) or vaccine cells (MCF10/DR7/CD80), and T cell activation quantified by measuring IFNγ release by ELISA. CD8-depleted PBMC contained < 2% $CD8^+$ T cells and 88.6% $CD4^+$ T cells at the start of priming. Data are representative of 3

independent experiments from 3 different healthy donors. ***B***, PBMC from healthy donor 100704 were either not depleted or depleted for $CD4^+$ or $CD8^+$ T cells prior to priming and boosting with Ii^- (MCF10/DR7/CD80) or Ii^+ (MCF10/DR7/CD80/Ii) cells. T cell activation was quantified by measuring IFNγ release. After depletion and before priming, CD4-depleted PBMC were <2% $CD4^+$ and 74.3% $CD8^+$; CD8-depleted PBMC were 78.2% $CD4^+$ and <1.5% $CD8^+$; not depleted PBMC were 44.2% $CD4^+$ and 38.3% $CD8^+$. Data are representative of 4 independent experiments using PBMC from 3 different healthy donors. ***C***, Supernatants from primed and boosted PMBC from panel B were tested for multiple cytokines by multiplex analysis.

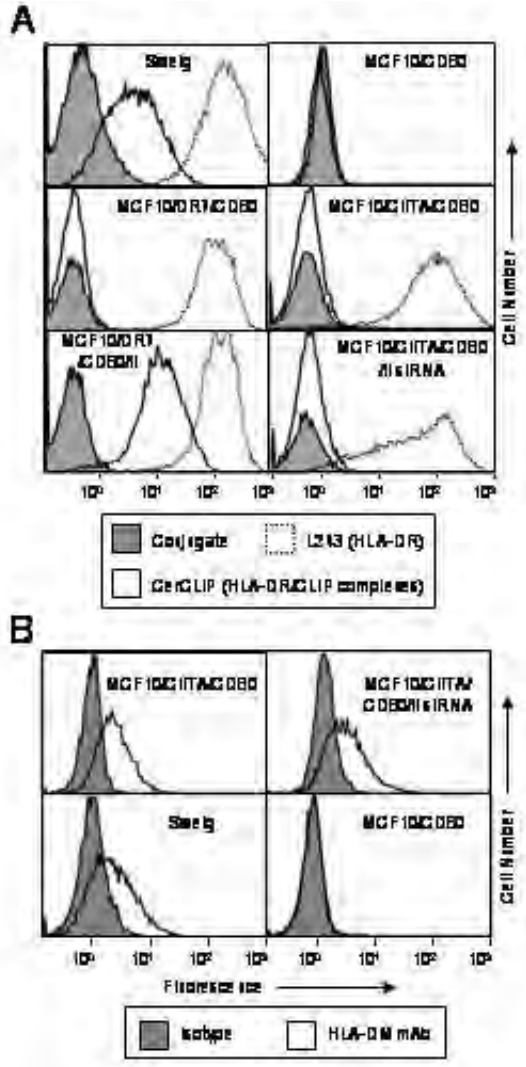

Fig. 3. MHC II vaccine cells do not express HLA-DR/CLIP complexes at the cell surface. Live MCF10 transductants were stained for cell surface HLA-DR/CLIP complexes (CerCLIP mAb), HLA-DR (L243) (A), or HLA-DM (B) and analyzed by flow cytometry.

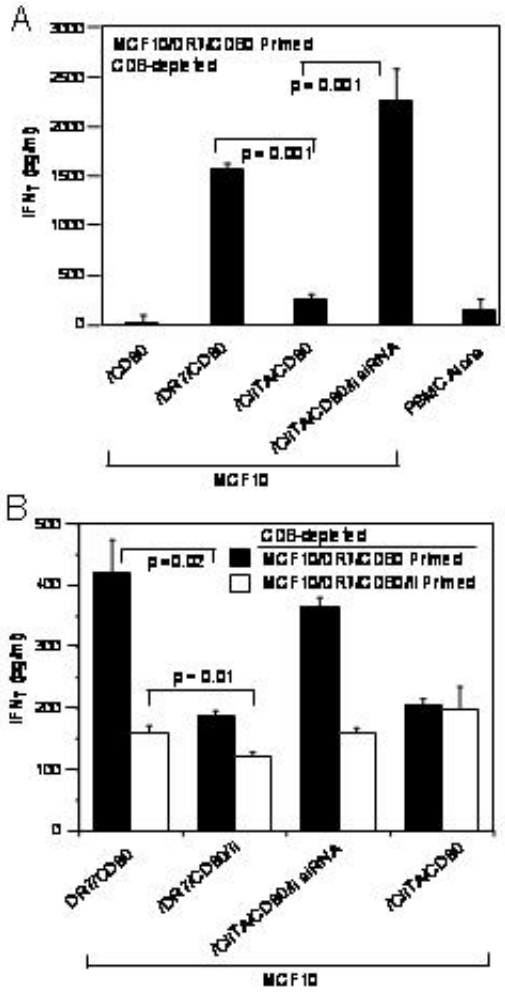

Fig. 4. HLA-DM expression does not affect $CD4^+$ T cell activation by MHC II breast cancer vaccine cells. ***A,*** PBMC from healthy donor 111504 were depleted for $CD8^+$ T cells, primed with Ii^- (MCF10/DR7/CD80) vaccine cells, and boosted with Ii^- (MCF10/DR7/CD80, MCF10/CIITA/CD80/Ii siRNA), Ii^+ (MCF10/CIITA/CD80, or control (CD80) transductants. T cell activation was quantified by measuring IFNγ secretion. After depletion and before priming PBMC were 87.5% $CD4^+$ and <2% $CD8^+$. ***B,*** PBMC from $DR7^+$ healthy donor 123104 were depleted for $CD8^+$ T cells, primed with Ii^- (MCF10/DR7/CD80) or Ii^+ (MCF10/DR7/CD80/Ii) cells and boosted with Ii^- or Ii^+ transductants as in A. After depletion and before priming PBMC were 80.7% $CD4^+$ and 4.9% $CD8^+$.

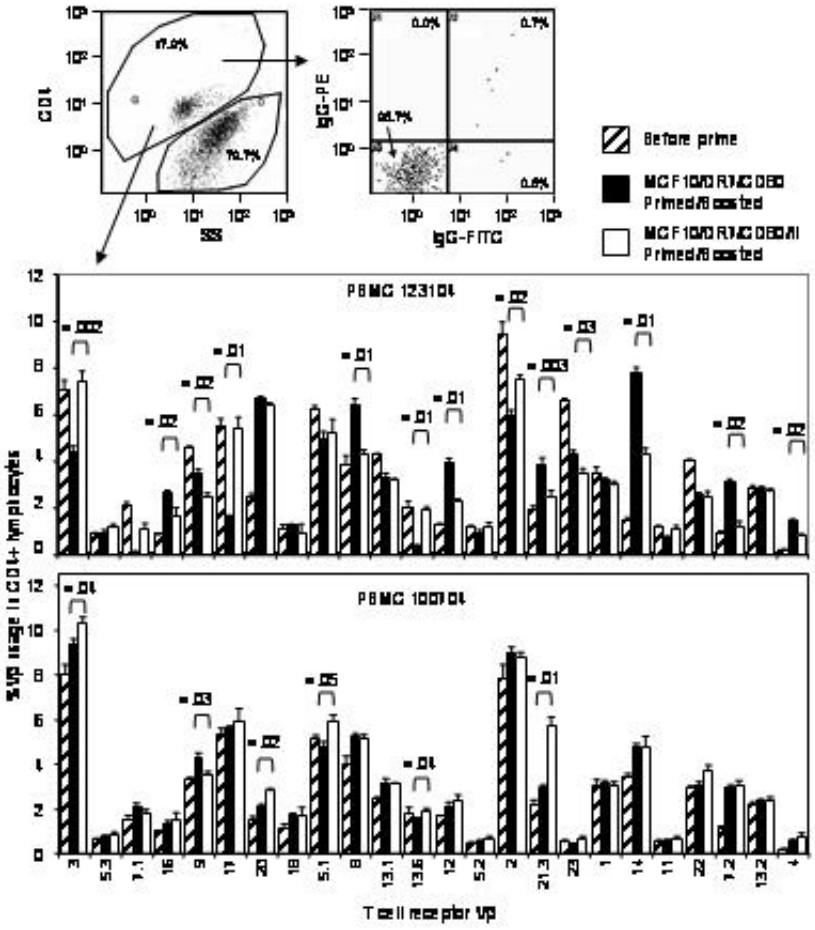

Fig. 5. Ii^- and Ii^+ MHC II breast cancer cells activate different repertoires of $CD4^+$ T cells. ***A,*** PBMC from healthy donor 123104 or 100704 were depleted for $CD8^+$ T cells, primed and boosted with Ii^- (MCF10/DR7/CD80) or Ii^+ (MCF10/DR7/CD80/Ii) cells and stained with mAb to CD4, mAb to TCR Vβ subtypes, or isotype control mAb. ***B,*** Gated $CD4^+$ T cells were analyzed for the percent of $CD4^+$ T cells expressing a given TCR Vβ. Data are the average of three independent experiments for each PBMC donor.

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Chapter 5

Discussion

Discussion:

The goal of this dissertation was to test our hypothesis that human tumor cells transduced with costimulatory and syngeneic MHC II molecules and lacking invariant chain activate a broad repertoire of $CD4^+$ T cells specific for tumor antigens. Previous studies showed that tumor cells genetically modified to express MHC II and the costimulatory molecules present endogenously derived antigens, activate $CD4^+$ T cells (1,2), and are effective therapeutic agents against tumor growth and metastasis in three mouse tumor models. The protective effect of these MHC II^+ tumor cells was deleted in the presence of the MHC II associated invariant chain (Ii) (3). I hypothesized that these MHC II vaccines had therapeutic efficacy because the absence of Ii enabled the presentation of novel tumor peptides that were not presented by Ii^+ cells. As a result, the Ii^- vaccine cells should activate T cells, which are not activated by professional APC, which are Ii^+, thereby inducing ant-tumor immunity. The studies described in this thesis support this hypothesis and demonstrate that the absence of Ii is a critical parameter for the activation of a distinct and broad repertoire of tumor-reactive $CD4^+$ T cells.

1. **MHC II is functional in the absence of Ii**

In order to support my hypothesis that MHC II presents novel peptides in the absence of Ii, I first had to prove that the lack of Ii did not affect normal MHC II function. To study the effect of Ii on MHC II, I genetically modified human tumor lines to express MHC II in the absence of Ii. I designed a retroviral expression vector that encodes the MHC II α and β subunits flanking an internal ribosomal entry site (IRES) (chapter 2). Coordinate synthesis of the α and β subunits overcomes the problems of alpha and beta chain instability and short half-life, and allows for efficient dimer formation. Human MHC II^- Ii^- mammary carcinoma cells

transduced with the retroviruses expressed normal levels of MHC II on their cell surface as shown by immuno-histochemistry and western analysis (Chapters 2 and 3). Stable cell surface MHC II expression in the absence of Ii has been observed in eight distinct human tumor cell lines with four different MHC II alleles (DR1, DR4, DR7, DR15) ((4,5) and unpublished data from Thompson, Bosch and Srivastava).

In addition to transducing single MHC II alleles, I have also generated Ii- vaccine cells by RNAi down-regulation of Ii in CIITA-transduced tumor cells (Chapter 3). For these studies I developed small hairpin RNA (siRNA) vectors which stably down regulate Ii over 95%. Up regulating MHC II via CIITA and down regulating Ii by RNAi did not reduce cell surface levels of MHC II (chapter 2 and 3). The absence of Ii did not affect function of the MHC II molecules since experiments with MHC II-defined HER2/neu peptides demonstrated that human MHC II molecules in both the single allele-transduced and the CIITA plus siRNA-transduced tumor cells were functional in the absence of Ii (4, 6-7) (Chapter 3). Therefore, whether MHC II^{-} Ii^{+} cells are transduced to express MHC II α and β subunits or whether Ii is down-regulated in MHC II^{+} Ii^{+} cells, MHC II is stable and functional in the absence of Ii in human tumor cell lines.

2. The absence of Ii probably results in the production of novel tumor peptides

In the absence of Ii MHC II may be free to bind peptides that normally would not be accessible to bind to MHC II in the presence of Ii. Because other researchers had found that Ii inhibited presentation of some endogenous antigens via MHC II (12, 8-11), Ostrand-Rosenberg et al. hypothesized that the absence of Ii would improve the ability of tumor cells to present a broader repertoire of immunogenic, endogenously expressed tumor antigens via MHC II. If Ii limits expression of endogenous antigens, then in the absence of Ii, novel endogenous antigens

may be presented that would not normally be presented via MHC II in the presence of Ii. This conclusion is supported by the studies of Muntasel et al, who demonstrated that most peptides bound to MHC II in the absence of Ii were from intracellular proteins and were unique to Ii^- cells (13). Identification of peptides bound to MHC II in the presence and absence of Ii also indicated that a broader repertoire of antigens was expressed in the absence of Ii (13). Therefore, the expression of MHC II in the absence of Ii may lead to the presentation of a broad array of novel tumor peptides that are not presented by profession APC that are Ii^+.

3. The absence of Ii may activate T cells that are not activated by Ii^+ APC.

If a distinct repertoire of tumor peptides is presented in the absence of Ii, then the Ii^- vaccine cells should activate some T cells that are not activated by Ii^+ APC. In order to assess whether different human T cell populations are activated by Ii^+ vs. Ii^- MHC II^+APC, I analyzed the percentages of $CD4^+$ T cells expressing specific TCR Vβ segment after activation with Ii^+ or Ii^- APC. $CD4^+$ T cells activated with Ii^- (MHC II^+ Ii^+ $CD80^+$) tumor cells expressed additional T cell subpopulations relative to T cells activated with Ii^+ (MHC II^+ Ii^- $CD80^+$) tumor cells. Although Ii^- tumor cells efficiently activated T cells primed by Ii^+ tumors, Ii^+ tumors did not efficiently activate T cells primed by Ii^- tumors (Chapter 4). Therefore, Ii^- APC probably present some of the same peptides as Ii^+ APC, but also present unique antigens not presented by Ii^+ APC. Our conclusion that Ii^- vaccine cells present unique peptides in addition to the peptides presented by Ii^+ APC is also supported by the studies described in chapter 3, showing that $CD4^+$ T cells specific for two defined tumor peptides are activated by Ii^+ or Ii^- APC. This conclusion is further supported by our observation that $CD4^+$ T cells activated by Ii^- APC were not efficiently

boosted by Ii^+ APC (chapter 4). Therefore, Ii^- tumors probably present many of the same peptides as Ii^+ tumors, and also present unique peptides which are not presented by the Ii^+ cells.

If Ii^- tumor cells express novel endogenous peptides not normally presented in the presence of Ii, then they have the potential to activate $CD4^+$ T cells with higher affinity than those $CD4^+$ T cells activated by Ii^+ APC. However, during normal T cell development, T cells with high affinity for self peptides are deleted. The novel peptides presented by Ii^- tumor cells are not presented during normal T cell development, so it is likely that an individual will retain $CD4^+$ T cells reactive with these novel peptides. Activation of such high affinity $CD4^+$ T cells may lead to an enhanced proliferative and more vigorous response to tumor. Our observation that Ii^- (MHC II^+ Ii^- $CD80^+$) tumor cells induce 1.5 to 2 times more T cell proliferation and significantly higher levels of IFNγ production by activated $CD4^+$ T cells, as compared to Ii^+ (MHC II^+ Ii^+ $CD80^+$) tumor cells supports this conclusion (chapter 4). Therefore, Ii^- vaccine cells are efficient activators of tumor-reactive $CD4^+$ T cells, consistent with our hypothesis that the absence of Ii promotes the activation of a more rigorous tumor-specific $CD4^+$ T cell response.

4. DC are needed for MHC II^+ Ii^- tumor vaccines to function and may present novel MHC II-peptides by cross-dressing.

Although our vaccines were originally designed to directly present antigen to T cells, a different mechanism may be responsible for T cell activation. Dolan et al have shown that DC are necessary for vaccine efficacy (14) and that they activate T cells by "cross-dressing," which involves the transfer of MHC-peptide complexes from tumor cells to DC (14, 15). Cross-presentation involves antigen uptake and processing by the DC and presentation of the processed

peptide on DC-encoded MHC molecules. In contrast, in cross-dressing, DC do not process antigen or present it bound to their own MHC molecules, but passively take up and present MHC-peptide complexes transferred from donor cells.

DC cross-dressing with MHC II-peptide from tumor vaccine cells may be enhanced by facilitating interactions between DC and tumor vaccine cells. If priming of naïve T cells occurs exclusively in lymph nodes, tumors which have not been shown to traffic to the lymph nodes would require cross-dressing or cross presentation through DC, or other cells which traffic to lymph nodes, in order to activate naïve T cells. Increasing the interaction between tumor vaccine and DC may enhance the transfer of MHC II-peptide complexes (Cross dressing).

5. How to improve vaccine efficacy.

If cross-dressing is the predominant mechanism of vaccine efficacy, then enhancing DC localization to vaccine cells may improve T cell activation. Tumor vaccine cells could be genetically modified to secrete DC localizing chemokines, such as CCL3 or CCL20, in order to bring greater numbers of DC into contact with tumor vaccine cells. Tumors secreting CCL3 and CCL20 are less tumorigenic, and reduced tumorigenicity correlates with the localization of DC to the tumor site (16). Granulocyte macrophage-colony-stimulating-factor (GM-CSF) also aids in localization and activation of phagocytic APC (17). Clinical trials using GM-CSF transduced tumor cells have met with some success (18). Therefore, vaccine cells expressing DC-attracting chemokines may be better donors of MHC-tumor peptide complexes and thereby facilitate cross-dressing and enhance tumor immunity.

Dolan et al have shown that for some antigens cross-dressing is more efficient than cross-presentation for activating T cells (14, 15). If cross-dressing is the predominant mechanism for

activating tumor-specific $CD4^+$ T cells, then Ii^- MHC II vaccine cells may be effective immunogens because they directly transfer to DC complexes of MHC molecules with novel tumor peptides. In contrast, wild type tumor cells either do not express MHC II or co-express MHC II and Ii, and therefore cannot cross-dress DC with novel peptides and can only activate $CD4^+$ T cells through cross-presentation. Since cross-presentation is known to be an efficient process for some antigens, and not for other antigens (15, 19-22), it is likely that optimal activation of $CD4^+$ T cells will occur through a combination of cross-presentation and cross-dressing. Therefore, efficacy of the MHC II vaccines may be due to their ability to activate $CD4^+$ T cells through both mechanisms.

6. $CD4^+$ T cells activated by tumor vaccine cells may facilitate activation of tumor specific $CD8^+$ T cells.

The MHC II vaccines are designed to activate $CD4^+$ T cells which will facilitate cytotoxicity by tumor-specific $CD8^+$ T cells and enhance long-term memory for protection against metastatic disease. Therefore, once activated, the $CD4^+$ T cells must only interact with DC or with $CD8^+$ T cells and there is no need for the activated $CD4^+$ T cells to react with patients' tumor cells. Since the MHC II vaccine cells express MHC I molecules, they also have the potential to activate $CD8^+$ T cells, provided the recipient and vaccine cells are matched for at least one MHC class I allele and the vaccine cells and the recipients' tumor share common tumor peptides. I envision that simultaneous binding of $CD4^+$ and $CD8^+$ T cells with the same vaccine cell (or DC if cross-dressing is occurring) would position helper and effector cells in very close proximity to each other, thereby maximizing the activation of effector and memory cells. MHC II vaccine-activated $CD8^+$ T cells would then kill patients' tumor cells and/or give rise to

memory cells for protection against latent metastatic disease. Therefore, optimal MHC II vaccines should not only be matched for an MHC II allele, but should also be matched to the recipient for an MHC I allele.

7. Ii shRNAs may be used to develop vaccines.

MHC II vaccines could potentially be developed from tumor cells that constitutively express MHC II by transduction with the Ii specific shRNA construct to down-regulate Ii expression. This strategy is supported by the studies of Chamuleau et al. who have shown that high levels of cell surface CLIP on MHC II^+ leukemias correlates with poor clinical outcome (23, 24). High CLIP expression is consistent with high levels of Ii expression, which I have demonstrated correlates with reduced T cell activation, a more limited repertoire of activated T cells (chapter 4), and in the mouse systems, no vaccine efficacy (25, 26, 11, 3) and reduced T cell activation. We are currently testing this hypothesis in collaboration with Chamuleau et al.. Patients' leukemic cells are being transfected with our Ii-shRNA vector and the resulting cells will be irradiated and given back to the patients as a "vaccine." Because large tumor burden often induces immune suppression which interferes with vaccine efficacy (27-29), patients will be give the Ii-shRNA vaccine when they are in regression. Since patients with leukemia typically relapse within 5 years of going into remission, treated patients will be followed to determine if relapse can be delayed or prevented. If the Ii shRNA modified leukemic cells are effective therapeutic agents, then patients may be in remission for extended periods.

This thesis has shown that MHC II^+ Ii^- tumor cells present a different peptide repertoire than do MHC II^+ Ii^+ tumor cells. I have supported my theory that MHC II^+ Ii^- tumor cells present novel peptides and that those peptides may induce better T cell activation than peptides presented in the presence of Ii. Therefore tumor vaccines expressing MHC II in the absence of Ii

may lead to better immune responses against tumor in vivo, than MHC II^+ tumor vaccines which express Ii. I have developed the tools necessary to genetically engineer tumor cells which express MHC II in the absence of Ii for use as clinical vaccines. Clinical trials are needed to further asses whether MHC II^+ Ii^- $CD80^+$ tumor vaccines will lead to increased survival in patients.

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Appendix I

shRNA expression vector for down regulation of mouse Ii.

shRNA expression vector for down regulation of mouse Ii.

Introduction

I previously developed effective human Ii shRNA expression vectors. Human and mouse Ii do not have sufficient homology to use the human shRNA vectors effectively in mouse cells. In order to study the effect of Ii on MHC II antigen processing and presentation in mouse cells new vectors needed to be developed. This appendix describes the design and creation of effective mouse Ii shRNA vectors.

Mouse Ii shRNA expression vector design: Complementary sequences in the coding region of the human Ii gene (GenBank NID NM_010545) were identified using the Ambion siRNA target finder search engine (Ambion, Inc. Austin, TX). Mouse siRNA sequence 30 was chosen because it has homology to human Ii sequence 4 shown to effectively down regulate human Ii (157). Other sequences were designed based on the absence of predicted secondary interactions using an RNA prediction algorithm (http://www.genebee.msu.su/services/rna2_reduced.html). Sequences with predicted open secondary structure and no homology to other known mouse mRNAs were chosen (54 and 64). Oligonucleotide siRNA expression cassettes were designed using Ambion's pSilencer™ Expression Vectors Insert Design Tool and modifying the predicted DNA cassette to have 5'-BamHI and 3'-EcoRI 'sticky ends' (http://www.ambion.com/techlib/misc/psilencer_converter.html). Oligonucleotide siRNA expression cassettes were prepared by MWG Biotech (High Point, NC) and inserted into the pSIREN Retro-Q vector (Clontech, Palo Alto, CA) according to the manufacturer's directions

(Clontech). The foreward (f) and reverse (r) primers were annealed and ligated to the linearized pSIREN-RetroQ vector with *BamHI* and *EcoRI* 'sticky ends'. SaI/CIITA cells were transduced with pSIREN-RetroQ/54 and tested for Ii and MHC II expression by immunohistochemistry using flow cytometry (Figure 1).

Cells: SaI and SaI/CIITA were handled as described (122).

Flow cytometry: tumor cells were stained for cell surface markers (MHC class II) or fixed and stained for Ii as described (122).

Results

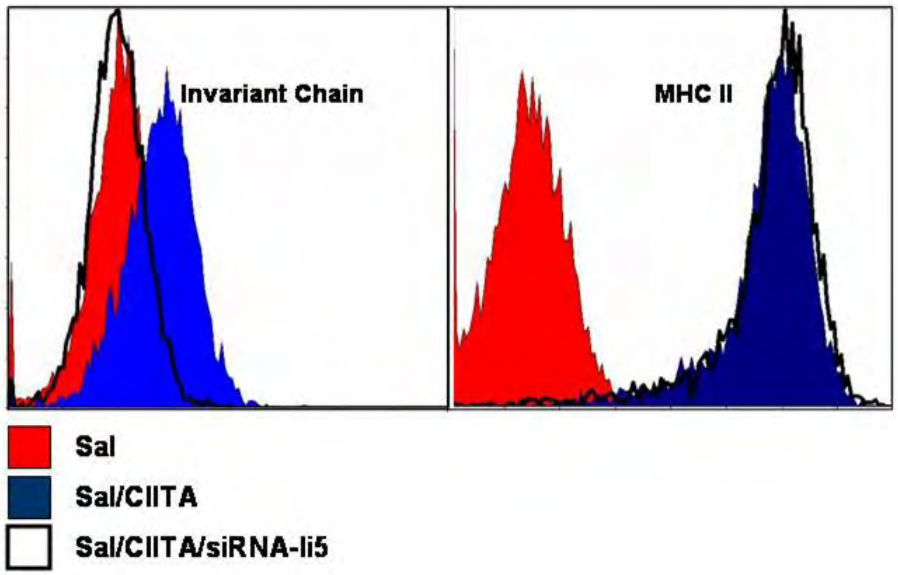
Mouse shRNA expression vectors successfully down regulate Ii. Mouse sarcoma cell line SaI/CIITA was transduced with the mouse Ii shRNA vector pSIREN Retro-Q-54. Table I shows the three different mouse Ii targets and the shRNA cassettes designed for insertion into the pSIREN-Retro-Q Retroviral shRNA expression vector. In order to test for down regulation of Ii by the shRNA vector and to verify that in the absence of Ii MHC II expression was not affected, SaI (Ii^- $MHCII^-$), SaI/CIITA (Ii^+ $MHCII^+$), and SaI/CIITA/ pSIREN Retro-Q-54 (Ii^- $MHCII^+$) were stained for Ii and MHC II expression. Cells transduced with the pSIREN Retro-Q-54 vector were down regulated for Ii expression by over 95% and had no significant change in expression levels of MHC II (figure 1). The other two vectors have yet to be tested.

Special thanks to Albert Forerro for aid in making shRNA constructs.

Table 1: mouse shRNA cassettes for insert into pSIREN Retro-Q vector

Target sequence 30	**AACCATGAGCAATTGCCCATA**
foreword sequence	5'-GATCCG CCATGAGCAATTGCCCATA TTCAAGAGA TATGGGCAATTGCTCATGGTT TTTTCTAGA-3'
reverse sequence	5'-AATTCCTCTAGAAAA AACCATGAGCAATTGCCCATA TCTCTTGAA TATGGGCAATTGCTCATGG CG-3'
Target sequence 54	**AAGCAGTGGCTCTTGTTTGAG**
foreword sequence	5'-GATCC GCAGTGGCTCTTGTTTGAG TTCAAGAGA CTCAAACAAGAGCCACTGCTT TTTTCTAGA-3'
reverse sequence	5'-AATTCCTCTAGAAAA AAGCAGTGGCTCTTGTTTGAG TCTCTTGAA CTCAAACAAGAGCCACTGCG-3'
Target sequence 64	**AAATGAAGTCAGAACAGAAGG**
foreword sequence	5'-GATCC ATGAAGTCAGAACAGAAGG TTCAAGAGA CCTTCTGTTCTGACTTCATTT TTTTCTAGA-3'
reverse sequence	5'-AATTCCTCTAGAAAA AAATGAAGTCAGAACAGAAGG TCTCTTGAA CCTTCTGTTCTGACTTCAT G-3'

Figure 1. SaI/CIITA transfected with mouse Ii siRNA expression vector pSIREN Retro-Q/54.1 down regulate Ii but do not affect MHC II expression levels.



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